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# PREFACE

We extend our grateful thanks to Dr. DeGraff who retires after ten years of devoted and successful efforts. Dr. Creger will assume the duties of Editor, and we welcome as Associate Editors Dr. E. William Hancock of the Stanford University School of Medicine, and Dr. Cecil Coggins of Harvard Medical School. As in past years the Annual Review of Medicine will try to provide critical reviews of particularly active areas of medical thought. Volume 24 is rich in papers drawn from such large areas as hormone and drug actions, and neoplasia, yet one also finds gems from smaller fields such as cytokinetics in skin disease, urethrovesical dysfunction, and endogenous carbon monoxide metabolism. We will continue to be fortunate indeed if our contributors of the next decade can meet the high standards set by their predecessors of the last decade. Finally, we also wish to thank Miss Beryl Daniel who has for so many years been our valuable Assistant Editor, and to welcome Mrs. Joanne Cuthbertson, our new Assistant Editor.

THE EDITORIAL COMMITTEE

# CONTENTS

CARDIOLOGY:

Genetics of Congenital Heart Disease, *Catherine A. Neill* . . . . 61
Viral Myocarditis and its Sequelae, *Walter H. Abelmann* . . . . 145
Primary Hyperlipoproteinemia in Infants, *Donald S. Fredrickson and Jan L. Breslow* . . . . . . . . . . . . . . 315

CLINICAL PHARMACOLOGY:

Drug Metabolism in the Newborn, *Margareta Eriksson and Sumner J. Yaffe* . . . . . . . . . . . . . . . . . . 29
Clinical Pharmacology of Drug Interactions, *Ilene H. Raisfeld* . . . 385

DERMATOLOGY:

Cytokinetics in Diseases of Epidermal Hyperplasia, *Gerald D. Weinstein and Jerry L. McCullough* . . . . . . . . . . 345

ENDOCRINOLOGY:

Blood Levels of Parathyroid Hormone in Disorders of Calcium Metabolism, *Eric Reiss and Janet M. Canterbury* . . . . . . 217
Insulin Receptors, *Bernard Desbuquois and Pedro Cuatrecasas*. . . 233
Human Prolactin, *Henry Friesen and Peter Hwang* . . . . . . 251
Recent Advances in Evaluation of Gonadotropic Hormones, *Judith L. Vaitukaitis and Griff T. Ross* . . . . . . . . . . 295
Normal and Abnormal Alpha Cell Function, *Roger H. Unger* . . 303
Thyrotropin Releasing Hormone: Secretion and Actions, *John F. Wilber* . . . . . . . . . . . . . . . . . 353

GASTROENTEROLOGY:

Action of Cholera Toxin on Fluid and Electrolyte Movement in the Small Intestine, *Geoffrey W. G. Sharp* . . . . . . . . . 19
Enterotoxic Diarrheal Syndromes, *Samuel B. Formal, H. L. Dupont, and R. B. Hornick* . . . . . . . . . . . . . . 103
Extraction of Insulin by Liver, *James B. Field* . . . . . . . 309

GENETICS:

Chromosome Identification, *Kurt Hirschhorn* . . . . . . . 67

HEMATOLOGY:

Platelet Function Tests in Disease, *Thomas B. Tschopp and Marjorie B. Zucker* . . . . . . . . . . . . . . . . . 1
Radiation and Drug Therapies, and Leukemia, *Abraham Potolsky and William P. Creger* . . . . . . . . . . . . . . 75
Current Concepts in the Treatment of Chronic Myelocytic Leukemia, *Joseph E. Sokal*. . . . . . . . . . . . . . . 281
Nature and Biological Activities of Degradation Products of Fibrinogen and Fibrin, *Hau C. Kwaan and Grant H. Barlow* . . . . 335

IMMUNOLOGY:

Immunologic Reconstitution, *W. D. Biggar, B. H. Park, and Robert A. Good* 135
Transfer Factor Therapy in Immune Deficiency States, *Alan S. Levin, Lynn E. Spitler, and H. Hugh Fudenberg* 175

INFECTIOUS DISEASE:

Preventive Antibiotic Management in Surgery, *John F. Burke* 289

NEPHROLOGY:

Urethrovesical Dysfunction and Infection, *Frank Hinman* 83
Renal Energy Metabolism and Sodium Reabsorption, *Peter R. Lewy, Antonio Quintanilla, Nathan W. Levin, and Richard H. Kessler* 365

NEUROLOGY:

The Lesch-Nyhan Syndrome, *William L. Nyhan* 41
Metabolism of Ketone Bodies by the Brain, *Louis Sokoloff* 271

OBSTETRICS:

Estimation of Gestational Age in the Fetal State, *John W. C. Johnson, Jr. and Gerard B. Odell* 165

ONCOLOGY:

Current Concepts in Treatment of Lymphomas with Special Reference to Hodgkin's Disease, *Leon Stutzman* 325

PSYCHIATRY:

Behavioral Aspects of Chromosomal Disorders, *Seymour Kessler and Rudolf H. Moos* 89
Mental Effects of L-Dopa, *Dennis L. Murphy* 209

PUBLIC HEALTH:

Methadone Treatment of Heroin Addiction, *Daniel X. Freedman and Edward C. Senay* 153

RESPIRATION:

Mechanics of Air Flow in Airway Obstruction, *A. C. DeGraff, Jr. and A. Bouhuys* 111
Endogenous Carbon Monoxide Metabolism, *Ronald F. Coburn* 241
REPRINT INFORMATION 419

INDEXES:

Author Index 421
Subject Index 443
Cumulative Index of Contributing Authors, Volumes 20 to 24 460
Cumulative Index of Chapter Titles, Volumes 20 to 24 462

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# PLATELET FUNCTION TESTS IN DISEASE[1] 7055

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Platelet function is assessed by many in vitro tests. Their physiologic basis, effects of pharmacologic agents, and alterations in disease have been reviewed elsewhere (1–7). Performance of the tests is crucial to their interpretation and will therefore be discussed first. Most animal studies are omitted as the platelet response differs from that of human platelets.

## METHODOLOGY

*Bleeding time.*—This test indicates the platelets' overall ability to build an impervious hemostatic plug in an injured blood vessel. The time is prolonged in patients with thrombocytopenia and qualitative platelet disorders or plasma abnormalities affecting platelet function. When the test is carefully standardized the time is prolonged by aspirin (8, 9) and varies with platelet age (10, 11).

*Clot retraction.*—This test measures the platelets' ability to condense the fibrin network and is affected by the platelet count, hematocrit, and fibrinogen concentration. When these variables are controlled, results are usually normal except in the rare congenital disorder called thrombasthenia.

*Technical difficulties.*—Other in vitro tests of platelet function require the use of an anticoagulant. Each type of the latter has an effect. Heparin can alter platelet function (12, 13); its action may be dose-dependent and evident at very low concentrations (14). EDTA sufficient to chelate plasma calcium ions prevents platelet aggregation and adhesion to glass. Citrate is usually used for in vitro study of platelet function. Blood is added to 1/9th its volume of 3.2–3.8% trisodium citrate. The plasma citrate concentration, which depends on the hematocrit as well as on the concentration of anticoagulant added, is 0.18–0.26 *M*. The calcium ion concentration is too low to permit thrombin evolution and is adequate but not optimal for platelet function (15).

Most platelet function tests are carried out on platelet-rich plasma (PRP) which is prepared by slow centrifugation of blood—a process which itself can affect platelet function (16, 17).

The platelets' response to stimuli depends in part on their plasmatic environ-

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ment. Differentiation is difficult because the function of platelets may be affected by the centrifugation necessary to separate them from plasma (13, 18).

The platelet count is often standardized to 200,000 or 300,000/mm$^3$ by adding platelet-poor plasma to PRP. Thrombocytopenic patients may have too few platelets to permit recording of aggregation. It is unsatisfactory to concentrate the platelets because of the difficulties cited above.

*ADP and platelet aggregation.*—Accumulation of platelets to form a plug in vivo is simulated in vitro by inducing platelet aggregation with one of several agents, most commonly ADP (adenosine diphosphate), epinephrine, collagen, or thrombin. Aggregation can be measured as a change in light transmission (or optical density) in PRP. Anticoagulant, pH, temperature, harvesting, and storage conditions of the PRP influence the results (13, 15, 19, 20). Although qualitative defects of platelet aggregation can be detected in this way, inter- and intra-individual variations detract from the method's value for establishing quantitative differences between patients.

ADP plays a key role in platelet function as it induces platelet adhesion to glass and platelet aggregation in the presence of ionized calcium. Much of the aggregation induced by other physiologic agents is caused by ADP derived from the platelets themselves. However, epinephrine and thrombin induce prompt "primary" aggregation which is not associated with ADP release. Under appropriate conditions, epinephrine and exogenous ADP induce a second wave of aggregation about one minute after the primary wave, caused by ADP freed by the release reaction (see below). Contact of platelets with collagen also induces aggregation associated with release; no primary aggregation is observed.

*Platelet release reactions.*—ADP, ATP (adenosine triphosphate), serotonin, and antiheparin activity (platelet factor 4) may be released during aggregation with ADP, epinephrine, connective tissue particles, or thrombin. Release by ADP and epinephrine requires a temperature exceeding 30°C and close platelet contact (13, 18). It is inhibited by aspirin, whose effect can last five days (8, 21, 22). Thrombin can in addition release amino acids, lysosomal enzymes, and metallic ions into the platelet environment—an effect called Release II (6). The physiologic importance of the release mechanism is shown by the prolonged bleeding time induced by aspirin (8, 9) and in patients with an absent or defective release reaction (23).

*Tests of platelet adhesiveness and retention.*—So-called platelet adhesion is estimated in one of two ways (24): Wright's method measures the fall in platelet count in citrated or heparinized blood rotated in a glass container under standard conditions, whereas Hellem's original method determines the percentage of platelets retained when citrated blood is passed through a glass-bead column. "Adhesiveness" is used to describe results based on Wright's test, and "retention" to describe results obtained with glass-bead columns.

Hematocrit, flow velocity, anticoagulant, timing and mixing procedures, age of the glass-bead column, and the type of plastic used influence results of both types of tests. Hellem's method has been modified by using citrated PRP with added ADP to induce retention or by using native or heparinized (17, 24, 25) whole blood. Results of three different tests using whole blood correlate well in normal subjects, but there was no correlation with results obtained using PRP with added ADP (26).

If ADP is not added, erythrocytes are necessary for platelet adhesion or retention. This was once thought to mean that the erythrocytes provide the ADP which makes the platelets sticky. They may do so with Wright's test since adhesion is prevented by destruction of ADP with an enzyme-substrate system (27). When measuring platelet retention with heparinized blood, however, incorporation of an enzyme that destroys ADP not only fails to inhibit retention but actually enhances it (17). In this case the erythrocytes probably promote platelet contact with the glass beads by their effect on flow (28, 29).

*Platelet contribution to coagulation; platelet factor 3.*—Platelet factor 3 (PF-3), the platelets' phospholipid contribution to the evolution of thrombin in blood, develops mainly during platelet aggregation. Unlike the substances discussed in the section on platelet release reaction, it remains largely bound to the platelets; its evolution does not require a temperature near 37°C, and is not inhibited by aspirin (30, 31). PF-3 activity in PRP can be induced with kaolin (32) or by prolonged incubation with ADP (33). The Stypven time (recalcification time with Russell's viper venom) is a more rapid measurement (34). PF-3 activity is difficult to reproduce in tests using washed platelets.

With inadequate platelet function or numbers, the so-called serum prothrombin time, or prothrombin consumption test, is abnormal although the whole-blood clotting time is usually normal.

Recent work (35) suggests that PF-3 is not the only contribution of platelets to blood coagulation, but a platelet function test based on this observation has not yet been devised.

*Electrophoretic mobility.*—Platelets have a negative electrostatic surface charge, and electrophoretic mobility of platelets has been measured with or without added ADP or norepinephrine (36).

*Platelet age and function.*—This subject has been well reviewed recently (37). The change in platelet function with age may hamper interpretation of platelet function tests in patients with compensated platelet destruction, since increased activity may simply reflect an increased proportion of young platelets. For example, this theory probably explains why platelet retention improves more rapidly than the platelet count after X-irradiation (38).

*Diurnal variation, epinephrine administration, and exercise.*—Platelet adhesiveness exhibits diurnal variation in most subjects; it is highest from about 1 to 3 PM. Adhesiveness is unaffected by eating, lipidemia, activity, or the phase of the menstrual cycle, and no sex- or age-related differences have been observed (39). A small (25 μg) subcutaneous injection of epinephrine causes a transitory rise (39). Aggregation induced by ADP at room temperature increases with the subject's age (40). About 8% of normal subjects show spontaneous platelet aggregation in vigorously stirred PRP (41).

Exercise does not alter platelet retention in citrated blood (42) but increases the platelet count (42, 43) and shortens the time for platelet aggregation measured in the Chandler loop, presumably because it accelerates coagulation (43).

## PLATELET FUNCTION TESTS IN DISEASE

### CONGENITAL DISORDERS DUE TO ABNORMAL PLATELETS

Congenital disorders due to abnormal platelets have been reviewed recently (44–46). Although the precise molecular defects are not yet known, several disorders have been fairly well characterized. A prolonged bleeding time and an abnormal serum prothrombin time are typical findings in all types.

*Thrombasthenia.*—In this rare, autosomal recessive disorder, platelets do not aggregate with ADP, epinephrine, collagen, or thrombin (47, 48). Clot retraction is absent or markedly impaired in most patients, and platelet fibrinogen is low (48). Absence of surface actomyosin may be responsible for the lack of ADP-induced aggregation (49). Some patients have a surprisingly mild hemorrhagic diathesis.

*Platelet release abnormalities.*—Such abnormalities resemble the defect induced by aspirin ingestion, and the hemorrhagic diathesis is usually mild. Aggregation induced by collagen is abnormal; by epinephrine, usually abnormal; and by ADP, normal (except at the critical concentration leading to the production of two phases in normal subjects). In some patients, the platelet granules responsible for storing the releasable serotonin, ADP, and ATP are present in greatly reduced numbers, causing so-called platelet storage pool disease (50). It occurs in some albinos (51, 52). In other subjects, there is a defect in functioning of the release mechanism (23).

*Bernard-Soulier giant platelet syndrome.*—This rare disease, autosomal and incompletely recessive, causes very severe bleeding. Since aggregation with the usual agents as well as the release reaction are normal, it is difficult to explain the prolonged bleeding time and abnormal prothrombin consumption, or to relate these to the platelets' failure to aggregate with bovine fibrinogen (53, 54). Abnormalities of electrophoretic mobility have also been described (55).

*Other qualitative platelet disorders.*—Various abnormalities of platelet function have been described in patients with Wiskott-Aldrich syndrome (56–58) and other forms of congenital, familial thrombocytopenia (58).

## CONGENITAL DISORDERS DUE TO PLASMA PROTEIN DEFICIENCIES

*Afibrinogenemia.*—Although washed platelets require fibrinogen for normal aggregation with ADP plus calcium ions, platelet aggregation in heparinized PRP is surprisingly normal (59, 60). With citrated PRP, the release reaction is more strikingly impaired than aggregation (60, 61).

*Von Willebrand's disease.*—This common hereditary hemorrhagic disorder is characterized by a prolonged bleeding time and low plasma antihemophilic factor (AHF, or Factor VIII) activity; it is inherited as an autosomal dominant trait with variable expressivity. Platelet retention is the only in vitro test of platelet function which is abnormal but whole blood must be used, rather than PRP plus ADP, and flow must be rapid (3, 24).

*Deficiency of Hageman Factor (Factor XII).*—At least some patients deficient in Hageman Factor have a prolonged bleeding time (62), abnormal platelet retention (63), and a poor or absent release reaction (64, 65).

## ACQUIRED DISORDERS OF PLATELET FUNCTION

*Acute illness, viral and infectious diseases.*—Acute myocardial infarction, pneumonia, venous thrombosis, and surgical operations make the platelets more sensitive to both ADP and norepinephrine when measured by electrophoretic mobility (66). The abnormality appears to be related to a change in the platelets since it cannot be conferred on normal platelets by adding plasma from affected patients. In contrast, patients with early syphilis show enhanced sensitivity to ADP but not to norepinephrine—a reaction characteristic of arterial disease. The abnormality could be conferred on normal platelets by adding the patient's plasma and disappeared with treatment. It is postulated that treponemal phospholipid is responsible (67).

Thrombocytopenia may develop after a number of viral diseases, possibly as a response to antigen-antibody complexes. Washed human platelets aggregate in the presence of a mixture of virus plus the serum of patients having antibody to the virus (68). Sixteen patients with severe infectious mononucleosis had some abnormality of platelet aggregation or release in the second or third week, without evidence of circulating platelet antibody or fibrin(ogen) degradation products (69).

Similarly, thrombocytopenia is common in septicemia, especially that due to Gram-negative organisms (70), even with little evidence of disseminated intravascular coagulation. Platelets aggregate in vitro with numerous types of bacteria but without killing them (71). This is postulated to be an important defense mechanism.

*Immunologic disorders.*—According to rather indirect evidence, platelets are important in the rejection episodes that may occur 4–50 days after renal transplantation (72, 73).

Natural heterologous antibodies induce biphasic aggregation of human platelets (74). A similar reaction can occur with an isologous antibody (75). Such antibodies can also inhibit aggregation induced by ADP or collagen rather than inducing aggregation (76). This type of response probably accounts for impaired platelet function in patients with chronic idiopathic thrombocytopenic purpura (77).

Several platelet function tests have been used to assess the presence of circulating antiplatelet antibodies in various types of thrombocytopenia. Among these are availability of PF-3 (78, 79), acid phosphatase (79), and liberation of radioactive serotonin (80).

*Surgical operations and shock.*—ADP- and collagen-induced platelet aggregation and release were reduced during and 1–3 hr after major surgery, returning to normal within 24 hr (81). Since the effect persisted after anesthesia had worn off, the agents used for this purpose were not considered responsible, nor were other drugs. Earlier investigators elsewhere as well as recent ones (82) reported an increase in platelet adhesiveness on the second postoperative day. The increase was abolished 10 min after intravenous administration of 1 or 10 units of heparin per kilogram. Electrophoretic mobility was not altered by surgical operations, but sensitivity to ADP and norepinephrine was enhanced (66).

Platelet aggregation was observed in the circulating blood of animals only when they were in severe hemorrhagic shock (83, 84). The increase in pulmonary vascular resistance occurring in animals with no circulating aggregates was attributed to vasoactive substances released from platelets (85).

*Disseminated intravascular coagulation (DIC); giant hemangioma.*—Thrombocytopenia, present in most patients with DIC, usually prevents study of platelet function. Changes in platelet function might be anticipated in DIC for several reasons. The youngest, most active platelets might be removed from the circulation by being incorporated into thrombi; the function of the remaining platelets would thus seem to be impaired because they would be older, on the average. Alternatively, circulating platelets may have been exposed to thrombin or to subendothelial material such as collagen without being incorporated into thrombi, with a resulting change in function. Finally, platelet function may be affected by the fibrinogen derivatives likely to circulate in patients with DIC. These are soluble complexes of fibrin monomer alone or with fibrin(ogen) degradation products.

Soluble fibrin monomer-degradation product complexes induce platelet aggregation because they can release platelet ADP (86, 87). Partially polymerized fibrin causes aggregation without release (88). The early compounds produced by the action of plasmin, representing relatively little degradation of the original fibrin(ogen) molecule, inhibit the effect of thrombin on platelets as well as on fibrinogen (89). Small, dialyzable, late products can inhibit platelet aggregation induced by ADP (87, 90). Since only relatively high concentrations of degradation products have in vitro effects, they probably do not play an important role in enhancing the excessive bleeding that may accompany DIC or fibrinonolysis.

Giant hemangioma with altered coagulation (Kasabach-Merritt syndrome) offers a particularly useful model of DIC since the condition changes very slowly, and the etiologic agent is the hemangioma itself, which is unlikely to exert other secondary effects on the blood (91, 92).

*Cyanotic heart disease.*—Children with cyanotic heart disease and polycythemia tend to develop thrombosis and excessive bleeding after surgery. Thrombocytopenia, increased bleeding time, and decreased levels of clotting factors are frequent findings. There is disagreement over whether these changes result from mild DIC (93–95).

*Liver disease.*—Among the causes of the abnormal hemostasis which often accompanies liver disease are reduced production of plasma clotting factors, enhanced fibrinolysis, DIC, and thrombocytopenia. Splenic sequestration in patients with an enlarged spleen is probably the principal cause of the low platelet count (96). Patients with hepatocellular disease usually show normal platelet survival which does not correlate with the shortened fibrinogen survival (97). Qualitative platelet defects are often present (96). The abnormal aggregation with ADP and thrombin has been attributed to fibrin(ogen) degradation products (98), although as mentioned in the section on DIC, they very probably do not reach a sufficiently high concentration. Development of PF-3 activity during incubation of PRP with ADP is impaired (99). Sodium deoxycholate causes platelet aggregation at concentrations of 0.43–1.7 m*M* (100). A low bilirubin concentration aggregated washed platelets, but 10 mg/100 ml were required in the presence of plasma (101). Chronic alcohol ingestion causes thrombocytopenia—presumably due to reversible inhibition of bone marrow; the platelet count is restored within about one week of abstinence from alcohol (102, 103). In vitro, 0.3% ethanol inhibits platelet aggregation due to release (104).

Patients with hepatomegalic forms of glycogen storage disease often suffer from excessive bleeding. Several platelet function tests including bleeding time and retention were abnormal in some but not all patients with Types I, III, and VI (105).

*Uremia.*—The bleeding tendency of uremic patients is associated with abnormalities of bleeding time, ADP-induced platelet aggregation, platelet retention, PF-3 availability, and clot retraction. The subject was recently reviewed (106). The fact that the bleeding increased and platelet retention decreased when levels of serum creatinine rose (107), and that dialysis improved hemostasis (106), points to a toxic substance in the plasma, but its identity is in dispute.

Guanidinosuccinic acid (GSA) levels of 0.1 mg/100 ml, achieved by adding uremic plasma or GSA to normal PRP, diminished PF-3 availability, whereas high concentrations of urea, creatinine, or guanidinoacetic acid had no such effect. GSA also inhibited the platelet release reaction induced by ADP, epinephrine, or collagen (108, 109). Others reported inhibition of PF-3 activity and secondary platelet aggregation by phenol (110) and especially by *p*-hydroxyphenylacetic acid at concentrations found in uremic plasma (111). Plasma levels of phenolic acids were correlated with PF-3 availability (111). The inhibitory effects of GSA and/or phenolic acids may have been potentiated by the imidazole buffer used by both investigative groups, since when used alone, imidazole inhibits platelet function (112).

Incubation of PRP with urea (200 mg/100 ml) inhibited platelet aggregation induced by ADP, epinephrine, or collagen (113). Other groups did not find that urea affected platelet function but they may not have incubated it long enough with PRP or used high enough concentrations (106). Infusion of urea into volunteers decreased platelet retention and, in a few, prolonged bleeding time (107). Platelets in uremic patients take up less serotonin than normal platelets, contain less, and store less (114).

*Diabetes.*—Since diabetics often have atherosclerosis and thrombosis, numerous attempts have been made to detect changes in platelet function that might contribute to these complications. Platelet retention in heparinized and citrated whole blood, and ADP-induced platelet aggregation were higher in diabetics than in age- and sex-matched controls (115–117). Elevated plasma glucose was ruled out as a cause since platelet retention was unchanged in normal subjects after intake of sugar (118). Even though ingestion of the same amount of glucose increased platelet adhesiveness, there was no correlation in time between the peak of plasma glucose and the adhesiveness test (119). Further evidence ruling out elevated glucose as a responsible factor is that reduction of blood sugar in diabetics by administering oral antidiabetic agents or insulin over 3–4 months did not lower platelet retention (117).

Plasma fibrinogen, Factor VIII, serum cholesterol, and plasma triglycerides are unlikely causes of increased platelet retention in diabetics (117, 120) because they are normal in potential diabetics (abnormal glucose test after cortisone) in whom platelet retention is abnormal (121).

When recalcified PRP from diabetics is rotated in plastic tubing (Chandler loop), the platelets aggregate more rapidly than normal, the more so if the subjects have vascular complications (122, 123). Platelets in diabetic PRP show normal first-phase aggregation with ADP (124, 125) but diminished disaggregation (124–126) with a significant negative correlation between the percentage of disaggregation and the corresponding serum cholesterol level (124). Platelets also undergo irreversible second-phase aggregation with lower concentrations of ADP than the platelets of age- and sex-matched controls, abnormalities which are even more prominent in patients with diabetic retinopathy (125, 126). Platelets from diabetics release more antiheparin activity than controls (127). When platelet electrophoretic mobility is measured, some diabetics exhibit the same enhanced sensitivity to ADP characteristic of atherosclerotic patients (128). Whether the platelets' increased responsiveness is due to their higher phospholipid content in diabetics (129) remains to be seen.

*Hyperlipemia.*—Patients with Type II hyperbetalipoproteinemia have a high incidence of arterial occlusion. Like patients with ischemic heart disease, they have a slightly higher than normal level of phospholipid per platelet, with a normal pattern, as well as increased PF-3 availability (130). Patients with Types II+IV or IV hyperlipoproteinemia who also had clinical evidence of atherosclerosis showed enhanced turnover of $^{51}$Cr-labeled platelets. Two patients with Type V disease associated with diabetes also had an increased platelet turnover (131).

When a sufficient quantity of a lipid emulsion containing soybean oil, lecithin, and glycerol was infused to cause a marked elevation of serum optical density, triglycerides, and fatty acids, platelet retention measured in citrated blood was significantly reduced, without affecting the platelet count or inducing hemolysis. In vitro addition of the emulsion increased platelet retention (132).

Betalipoprotein added in vitro accelerated ADP- and thrombin-induced aggregation and ADP-induced retention of platelets in PRP (133). The betalipoprotein from rabbits on a high cholesterol diet was more active than that from normal rabbits (134).

Study of the platelets of pigs on a high fat diet revealed changes in the type of fatty acid in the phospholipids, rather than the type of phospholipid or percentage of various types of lipid (135). Similarly, the platelets of rats on a thrombogenic, high-fat diet showed a higher ratio of saturated and mono-unsaturated to polyunsaturated fatty acids. This ratio was also increased in patients who had suffered a myocardial infarction; in both rats and patients, it was associated with an increased susceptibility to thrombin- but not ADP-induced platelet aggregation (136). In both species, PF-3 activity was also proportional to the higher serum cholesterol levels (137).

*Atherosclerosis and thrombosis.*—The abnormalities in blood platelets and clotting factors responsible for hemorrhagic diatheses have been well characterized both functionally and biochemically, but little is known about a problem which is more important from the viewpoint of frequency and consequences—why some individuals are likely to have a thrombotic episode. Significant differences, to be described, have been noted between patient and control groups but too much overlap prevents identification of abnormal individuals by the tests employed.

Platelet retention was substantially increased in the first 48 hr after myocardial infarction (MI) (116, 138–140), and decreased (116) or returned to normal values 4 weeks after the event (139). In contrast, one group noted a decreased number of adhesive platelets (141). Platelet retention was also enhanced, compared to age- and sex-matched controls, in patients with acute thrombophlebitis (116, 138), recurrent venous thrombosis (142), or occlusive vascular disease (138). These findings suggest that enhanced platelet retention is linked to acute tissue destruction, but no correlation was found between the number and severity of strokes in patients with cerebrovascular disease and platelet adhesiveness, which was increased compared to controls (143).

In acute MI, platelets in citrated blood showed more than a normal amount of pseudopods and clumping when spread on films and viewed with an electron microscope (144). PRP did not show the changes in optical density that usually denote platelet shape change after the addition of ADP (145, 146). Plateets aggregated more readily than normal at room temperature (40) as well as at 37°C. Thus, ADP at 1.4 $\mu M$ induced second-phase aggregation in 20 of 24 patients, but in only 2 of 14 normal subjects (146), agreeing with similar observations of others (147), and in patients with cerebrovascular disease (148, 149). Platelets of patients with occlusive vascular disease disaggregated less than controls after the addition of 1.7 $\mu M$ ADP (150).

In patients 2–12 months after MI, platelet adhesiveness and retention were normal (151–153). Controls and patients showed no differences in platelet adhesiveness in venous or arterial blood; blood collected from controls after applying stasis showed an increase in platelet adhesiveness, but this was not true of study patients (154).

In heparinized PRP, various parameters of platelet aggregation induced by ADP, serotonin, epinephrine, and collagen were increased in patients with old as well as acute MI (145).

Another type of platelet aggregation test (PAT) (155) revealed abnormalities in atherosclerotic patients. This test consists of rotating citrated PRP in a siliconized glass bulb at 37°C for 10 min. Diluted PRP is then placed on a glass slide for 30 min, fixed and checked for number and size of aggregates under the light microscope. Abnormal platelet aggregation was seen in 92% of atherosclerotic patients, which depended on a nondialyzable plasma factor. This factor, adsorbed by $BaSO_4$ and similar compounds, was heat-stable and not neutralized by heparin. No correlation was found between the increased PAT and cholesterol or free fatty acid concentration in the patient's blood (155).

Various parameters of platelet aggregation induced by ADP or collagen and assessed with the nephelometric method and the PAT were recently compared in patients with progressive thrombotic obliteration of a peripheral artery (156). Patients and controls showed no difference in nephelometric aggregation induced by ADP or collagen, whereas abnormal PAT tests were encountered three times as often in the patients. There was no correlation between results of the two methods.

Enhanced ADP-induced aggregation and a drop in adhesive platelets has been reported in patients with angina pectoris immediately after taking a Master's exercise test; neither parameter was altered in normal subjects under these conditions (157, 158). A double-blind crossover trial indicated prevention or lesser effects in subjects who had ingested 1 g pyridinolcarbamate 3 hr earlier. Ingestion of the drug itself did not affect platelet aggregation.

It has been reported that electrophoretic mobility of platelets in PRP from atherosclerotic patients was increased by only one tenth as high a concentration of ADP as was required with normal PRP (128). The sensitivity to norepinephrine was not altered. These findings have been confirmed (159). The abnormal sensitivity could be conferred on normal platelets by fresh plasma from abnormal subjects. Ingenious experimentation and careful reasoning indicated that the sensitivity was caused by lecithin, possibly free in the plasma, acting in the presence of ADP, which was released during centrifugation (128, 160) or was added (161).

*Oral contraceptives.*—According to some investigators (162, 163) women on oral contraceptive pills containing synthetic estrogen and progesterone have a higher incidence of thromboembolism than women not on the pill, a view not shared by all (164). Poller (165–168) noted abnormally rapid aggregation of platelets in recalcified PRP rotated in a Chandler loop, but a more marked effect was noted in the third trimester of pregnancy. This method probably measures aggregation induced by thrombin, and its enhancement may simply reflect the accelerated evolution of thrombin and/or fibrin. Thus, fibrin formation starts earlier and develops more rapidly when assessed by the thrombelastograph (169, 170), and as in late pregnancy Factors VII and X are increased (166, 171). However, the shortening of aggregation time can precede the rise in Factors VII and X (168). ADP-induced aggregation has been described as normal (165) or enhanced (172). Most authors did not report an increase in platelet adhesiveness (173, 174) or in platelet retention measured in whole citrated blood (175) or PRP with added ADP (172, 176), although one group observed increased platelet adhesiveness which did not correlate with increased activity in postheparin lipolysis (177). An increase in platelet sensitivity to ADP, as measured by electrophoretic mobility, was observed by one group (178). The effect developed within four days and disappeared during the week between cycles. As in arterial disease, the effect was ascribed to an abnormality in plasma lecithin. An enhanced response of platelet adhesiveness to a low molecular weight basic protein (EF) derived from brain has also been reported (173, 179).

When progesterone alone was administered, platelet aggregation in the Chandler loop was not accelerated after the first six months (165) but some shortening was noted after 1–2 years (167, 168). ADP-induced changes in electrophoretic mobility were not altered (178).

When natural or synthetic estrogens were given to normal males, platelet adhesiveness was unaltered but ADP- and norepinephrine-induced platelet aggregation were enhanced. Only the synthetic compounds altered the electrophoretic response to ADP, as did estrogen-containing oral contraceptives (180).

*Multiple sclerosis.*—A review of earlier studies indicated increased platelet adhesiveness in multiple sclerosis (181). Others showed that the chronic phase is characterized by increased platelet sensitivity to ADP but not to norepinephrine, as measured by electrophoretic mobility (181). This response resembles that of patients with arterial disease. Sensitivity to both ADP and norepinephrine is enhanced in the acute phase as in various other acute illnesses. The agent conferring increased sensitivity to ADP is in the plasma but, unlike the agent in the plasma of patients with arterial disease, it is labile. Platelet adhesiveness is markedly enhanced by EF, the protein from brain, in patients with active multiple sclerosis but not in normal subjects or patients in a quiescent stage of the disease. Sensitivity to EF is conferred on normal platelets by plasma of patients with active disease (182). Changes in platelet response are strikingly similar in multiple sclerosis and after use of oral contraceptives, whether measured with EF or as altered sensitivity of electrophoretic mobility to ADP (179).

*Myeloproliferative disorders.*—Bleeding has long been known to be a problem in leukemia and other myeloproliferative disorders but its cause is poorly understood. The presence of thrombocytopenia or thrombocytosis often hinders accurate measurement of platelet function. Deficiencies in procoagulant plasma factors are common (183) and may affect platelet function. Increased ADP consumption and adenosine deaminase activity in leukemic plasma (184, 185) may account for abnormal platelet aggregation. Such drugs as vincristine may affect platelet ultramorphology and release (186).

Essential thrombocythemia has been associated with variable bleeding times, decreased platelet retention, reduced PF-3 availability, no epinephrine-induced aggregation, little if any ADP-induced aggregation, and normal collagen-induced aggregation (187–191). In chronic granulocytic leukemia, platelet aggregation by ADP, epinephrine, and collagen was diminished (192). Others found normal values for ADP, epinephrine, and collagen-induced aggregation in such patients (191). The release of PF-4 (antiheparin activity) varied (193).

Still to be resolved are whether these disparate findings are due to inadequate classification of myeloproliferative disorders or whether they reflect different abnormalities in different individuals.

*Multiple myeloma and macroglobulinemia.*—Impaired platelet function is a common finding in patients with multiple myeloma or macroglobulinemia and high levels of abnormal proteins (194, 195). Malfunction is reflected by a bleeding time prolonged out of proportion to the degree of thrombocytopenia. Clinical bleeding is associated with a prolonged bleeding time and abnormal platelet adhesiveness (194). The test results improve and hemorrhage ceases when the protein concentration is reduced by plasmapheresis (195, 196). Platelet aggregation may be decreased (195, 197) but is occasionally increased (198). PF-3 activity is not increased when assessed with washed platelets (194) but may be abnormal when PRP is used (197).

A few patients with multiple myeloma have abnormal transparent clots that fail to retract, as well as a long thrombin clotting time. These abnormalities are ascribed to abnormal fibrin polymerization rather than poor platelet function and are not necessarily associated with a hemorrhagic diathesis (199).

*Glomerulonephritis.*—A number of children with active renal disease exhibited enhanced platelet aggregation in response to collagen and ADP. Platelet function was normal when the disease was clinically inactive. The degree of abnormality correlated with the degree of proteinuria and the plasma fibrinogen-to-albumin ratio but not with the hematocrit, the presence of soluble fibrin polymers in the plasma, or the level of the third component of complement (200).

*Hypertension.*—Platelet adhesiveness (heparinized blood) was found to be high in hypertensive patients (201).

*Osteogenesis imperfecta.*—Some patients with this condition showed decreased platelet retention, aggregation, and PF-3 release; fewer had a prolonged bleeding time; only rarely was there evidence of excessive bleeding (202).

*Scurvy.*—Platelet adhesiveness and ADP-induced aggregation are impaired in human scurvy (203, 204) as well as in guinea pigs with induced scurvy (205, 206).

*Homocystinuria.*—Diminished platelet aggregation was observed with ADP but responses with collagen and epinephrine were normal. The abnormality with ADP was reported to be improved by administration of pyridoxine (207). Two patients exhibited abnormally high sensitivity to ADP when measured by electrophoretic mobility (128).

*Prostatic carcinoma.*—Patients with this disease had a higher platelet count and a greater number of adhesive platelets than those with benign prostatic disease. Platelet adhesiveness expressed in per cent however was the same in both groups (208).

*Hereditary hemorrhagic telangiectasia.*—This is not usually considered a generalized disorder of hemostasis, but in one study many patients showed a decrease in in vivo platelet adhesiveness (209), and in another, two patients had a prolonged bleeding time with markedly decreased platelet retention (210).

*Down's syndrome and childhood schizophrenia.*—Since platelets can concentrate serotonin and store it in special granules, it is significant that the platelets of patients with Down's syndrome are low in serotonin (211, 212) and monoamine oxidase (211, 213). There is a disagreement as to whether serotonin uptake is normal (213) or reduced (211).

Platelet serotonin uptake is normal in childhood schizophrenia (214).

## SUMMARY

Abnormal platelet function may result from congenital or acquired disturbances in the platelets themselves or their plasma milieu in patients with hemorrhage or thrombosis. Although standardization is difficult and results overlap between patient groups, certain tests are abnormal in patients with diabetes and atherosclerosis, and in women on oral contraceptives, all of whom have an increased tendency toward thrombosis. These findings suggest a possible breakthrough in this important public health problem.

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# ACTION OF CHOLERA TOXIN ON FLUID AND ELECTROLYTE MOVEMENT IN THE SMALL INTESTINE

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Excellent reviews are available that provide a comprehensive account of cholera including patient care, the history of research, and the actions of cholera toxin in man and in experimental animals (1–5). Rapid advances have been made recently in understanding the action of cholera toxin at the cellular level. Thus this account will deal mainly with two aspects of the topic: 1. the nature of the effects of the toxin on water and electrolyte movement, and 2. the action of cholera toxin in stimulating adenyl cyclase.

The molecular mechanism by which cholera toxin produces massive loss of water and electrolytes is unknown. However, an increase in net secretion of approximately isotonic fluid occurs throughout the length of the small intestine. The fecal fluid remains essentially isotonic, has raised bicarbonate and potassium concentrations relative to plasma, and contains little protein. Bicarbonate concentration in different areas of the small intestine varies, being lower than plasma in the duodenal fluid and higher than plasma in the ileum (6–8). These findings occur in association with physiological studies and with light and electron microscopic studies showing an intact and apparently undamaged mucosa (9–11). Thus, in cholera, secretory transport processes occur to such an extent that reabsorptive capacity is exceeded and diarrhea results. The relative contributions of increased active ion secretion and decreased active ion reabsorption to this diarrhea are still to be defined.

The cholera exotoxin responsible for these effects has been extensively purified (12–15) and recently crystallized (16). It is a protein with a molecular weight of about 84,000 and is both heat and acid labile. The toxin has the ability to induce intestinal fluid production after brief application to the mucosa even if followed by extensive washing (1). Thus, in all likelihood, the toxin is rapidly bound to the luminal surface of the intestinal cells. Because little is known of the uptake of macromolecules in the intestine, one can only speculate about the mechanism. However, an interesting binding interaction occurs between toxin and gangliosides which could be involved in the rapid uptake (17).

Perhaps the most important observation for our understanding of the effects of cholera toxin on ion transport is that of Field, Plotkin & Silen (18), which suggested that chloride and possibly bicarbonate could be actively secreted by rabbit ileum in response to adenosine-3′,5′-monophosphate (cyclic AMP). In this study, segments of distal ileum were stripped of the outer mesothelium and the two principal muscle layers so that the remaining mucosal epithelial layer could be mounted as a membrane between the two halves of a lucite chamber. Both surfaces were bathed in oxygenated Ringer's solution and short-circuit current was measured, by the technique of Ussing & Zerahn (19), as an indicator of net ion flux. Addition of cyclic AMP to the serosal bathing medium increased the short-circuit current such that either cations were stimulated to move from the mucosal medium to the serosal medium or anions were stimulated to move from serosa to mucosa. By the use of sodium-free, chloride-free, or bicarbonate-free Ringer's solutions it was possible to show that increases in current, although smaller than previously, occurred in the absence of sodium and in the absence of chloride, but not in the absence of both chloride and bicarbonate. This study indicated a possible action of cholera toxin to stimulate anion secretory processes into the lumen of the intestine in excess of reabsorptive capacity. When a crude preparation of cholera toxin was added to the luminal surface of the stripped ileum, the short-circuit current started to increase after a 30 to 45 min latent period and reached a peak plateau 90 min later (20). After elevation by cholera toxin the short-circuit current was only slightly affected by the further addition of cyclic AMP or theophylline (which increases cyclic AMP concentration by inhibition of phosphodiesterase). Other studies also showed an association of the action of cholera toxin with the action of cyclic AMP. For instance, Pierce et al demonstrated that prostaglandins and theophylline could cause fluid production in the small intestine of dogs when infused into the mesenteric artery (21). Similarly, lipolysis in fat cells (22, 23) and glycogenolysis in platelets (24), both of which are stimulated by cyclic AMP, were both stimulated by treatment with cholera toxin. Direct evidence of a relationship between intracellular cyclic AMP and cholera toxin in intestine came from the work of Schafer et al (25). Working on dog intestine, they found little effect of the cholera toxin during the first hour after administration. In the second hour, however, cyclic AMP concentrations in the mucosal epithelial cells began to rise and reached levels three or four times higher than control values at 4 to 5 hr. Reports that adenyl cyclase activity was elevated by cholera toxin in rabbits, guinea pig, and man confirmed the hypothesis and identified the key enzyme (26–30).

The observation of Field et al that short-circuit current is increased by cholera toxin (18, 20) implies that the net effect is either anion movement from serosa to mucosa or cation movement from mucosa to serosa. As increased cation movement from mucosa to serosa would result in increased absorption from the lumen (the reverse of diarrhea), the probable explanation is increased anion movement from body to lumen. As the short-circuit current demonstrates only net effects, the possibility exists that more than one change in electrolyte handling may be occurring simultaneously. It may be significant also that the changes in short-circuit current are less with purified cholera toxin than with the crude toxin. Thus anion movement from serosa to mucosa could occur, as well as coupled cation/anion movement from serosa to mucosa and decreased cation movement from mucosa to serosa. Only the net effect of changes such as these will be represented by the short-circuit current. Elucidation of these effects will prove to be a difficult undertaking, compounded by the fact that passive movement of ions in the intestine exceeds the active movement. That is, the ratio of active to passive movement is low, and one hopes to detect changes in the small component of transport against a high background. Although at first sight in vitro studies would appear the most promising line of attack, the difficulty of maintaining viability of the tissue in vitro and the possible removal of physiological stimuli to normal function must be considered. Electrolyte flux measurements based on human or whole animal studies in vivo are difficult to interpret precisely. For instance, it is well recognized that differences in water and electrolyte movement across the intestine can be caused by differences in hydration of the body (31–33). Thus, patient studies involving intravenous fluid replacement will show variability due to the degree of under- or over-hydration achieved. Technical problems, lack of firm knowledge of compartmentalization, and the presence of a large unstirred layer in the brush borders and crypts in association with mucus must be considered. For many reasons, therefore, in vitro studies may be the information source most likely to reveal the role of cholera toxin in fluid production in the small intestine. Perhaps because of all the difficulties, the literature is replete with conflicting data and conclusions. For example, with respect to sodium handling, there is evidence for decreased lumen to plasma sodium movement (34), for decreased lumen to plasma and plasma to lumen sodium movement (35) with the former being the greater change, for no change in unidirectional lumen to plasma sodium flux (36, 37), and, of course, for increased plasma to lumen sodium movement (36, 38). How then can we resolve these problems? The author does not believe that a valid solution is available at this time. Perhaps it is better not to try to resolve differences in results when different techniques, luminal flow rates, and toxin preparations, not to mention species, are involved, because the differences in results may well be due to the different techniques used. Thus, only a few selected references have been mentioned here; all but one are in vitro studies.

Moore et al have assessed the effect of cholera toxin on water and electrolyte transport in the dog ileum by a recirculation technique (39). Water absorption decreased from the second hour after challenge and was changed to fluid secretion at four hours. Potential difference across the ileum changed at the third hour from a small potential (2–3 mV) lumen positive to a potential with the lumen negative. These changes were associated with increased potassium and bicarbonate and decreased chloride concentrations in the recirculation fluid. It was concluded from these studies that cholera toxin caused active bicarbonate and chloride secretion. Accumulation of sodium ions in the lumen was considered to be passive. Secretion of potassium was thought to be partly passive and partly active—this is because the potassium concentration in luminal fluid was higher than one would anticipate from a consideration of the Nernst equation.

In experiments on isolated ileum from rabbit and human, Field et al (5, 20) and Rhode (40) have concluded that chloride secretion is the major response to cholera toxin. When short-circuit current and the fluxes of sodium and chloride in a preparation of isolated rabbit ileum were measured simultaneously, sodium

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and chloride fluxes from mucosa to serosa were greater than fluxes from serosa to mucosa; thus reabsorption was occurring under control conditions. The sodium and chloride fluxes, however, did not account completely for the short-circuit current. Thus, another ion flux was present which represented either a cation from lumen to body or anions from body to lumen; it is believed to be a body to lumen bicarbonate flux. With this system studies have been performed using cyclic AMP, theophylline, and cholera toxin. All these agents increased the short-circuit current and reduced the net sodium flux to zero. The direction of chloride flux was reversed so that chloride secretion resulted. The unidentified residual flux was not altered by these treatments. The effect of cholera toxin was qualitatively similar to the effect of cyclic AMP and theophylline, except that the changes with cyclic AMP occurred promptly and those due to cholera toxin only after a latent period. Cholera toxin began to stimulate short-circuit current after about 45 min and rose to a plateau an hour or two later. It was concluded that cholera toxin and cyclic AMP stimulate chloride secretion while also inhibiting sodium reabsorption. The inhibition of sodium reabsorption occurs only in the substrate independent pathway, as glucose or amino acids will still cause the expected increase of short-circuit current and increased sodium reabsorption. The inhibition of sodium reabsorption is controversial in that it could be due to a completely independent system and effect or to a coupled sodium-chloride pump from body to lumen. Unidirectional flux studies do not support the latter possibility in Field's experiments while a separate effect completely unrelated to chloride also seems unlikely. For example, when bicarbonate or chloride were replaced by sulfate so that cyclic AMP could not exert its effect on chloride transport, an independent effect on sodium transport to inhibit mucosa to serosa movement should have resulted in a decrease in short-circuit current. This was not the case. Further studies are required to decide whether a coupled sodium-chloride pump operating from plasma to lumen is stimulated by cholera toxin as suggested by Powell, Binder & Curran (41).

As an increase in cyclic AMP can be achieved by inhibition of phosphodiesterase as well as by a stimulation of adenyl cyclase, measurements have been made of the activity of both these enzymes under control and toxin-treated conditions (26, 27). While markedly increased activity of adenyl cyclase occurred, no changes were detected in phosphodiesterase activity. The adenyl cyclase activity was increased to very high levels when large doses of toxin were used. The toxin is known to be heat sensitive and boiled toxin was inactive. Cholera toxin increased adenyl cyclase activity in the lower ileum, the jejunum, and the duodenum, a finding in agreement with an effect of the toxin on the whole small intestine. Increased enzyme activity was detected only 15 min after the application of toxin and the activity then increased progressively, a sequence of events in general accord with the time course of effect of cholera toxin on electrolyte flux changes. In a more precise study on the temporal relationship of adenyl cyclase activity and flux changes, dogs given a 10 min pulsed application of cholera toxin showed a close relationship between sodium and water movement across the intestine and the stimulation of adenyl cyclase by the toxin (42). It was found that

90 min after the application of cholera toxin the normal reabsorptive activity of the intestine had changed to a secretory function which corresponded with an increase in adenyl cyclase activity in the mucosal epithelial cells. Adenyl cyclase activity and the secretion of fluid increased to maximum values at about 3 hr. Cyclase activity and fluid production were still elevated at 24 hr and had returned to normal by 48 hr. Thus a close correspondence between adenyl cyclase activity and fluid production was found.

Although the molecular interaction by which cholera toxin increases the activity of adenyl cyclase is not known, some characteristics of its action have been examined in the hope that a key to the mechanism would be uncovered (26, 27, 30). The main feature of the effect of cholera toxin is a large increase of basal enzyme activity without a proportional increase in the level of fluoride activation. Fluoride universally stimulates adenyl cyclase from mammalian tissues to near maximal levels. The lack of a proportional increase with fluoride strongly suggests that no synthesis of adenyl cyclase occurs in toxin-treated tissue because fluoride activation should increase in direct proportion to the increase in amount of enzyme. The possibility that an activating factor is released into the cytoplasm or the idea that cholera toxin is itself a soluble adenyl cyclase are both unlikely because the increased enzyme activity after exposure to cholera toxin can be observed in well-washed membrane preparations (27). In fact the latter suggests that a relatively permanent change in the enzyme has occurred. This may be due to the binding of toxin or a fragment of toxin by the enzyme. It is also possible that the toxin is itself an enzyme which brings about a permanent change in membrane structure to cause an increase in the activity of adenyl cyclase.

The conditions for the assay of adenyl cyclase activity in mucosal epithelial cells of rabbit small intestine have been defined in the basal state and after the enzyme has been activated by prior exposure of the tissue to cholera toxin. Optimal conditions for activity were the same with regard to magnesium concentration, pH, and ATP concentration. Examination of the activity of adenyl cyclase at different ATP concentrations revealed that cholera toxin stimulated the activity by an increase in the $V_{max}$ without any change in $K_m$. No evidence of an allosteric confirmational change was detected. Calcium was inhibitory; manganese could replace magnesium at low concentration but was inhibitory at high concentration. Again, no difference was observed between the effects of these agents on the basal enzyme activity and on the enzyme activated by cholera toxin (Sharp et al, unpublished). Thus, the increased activity of intestinal adenyl cyclase after exposure to cholera toxin appears to be achieved without any obvious change in the characteristics of the enzyme except for the possible enhancement of responsiveness to prostaglandin $E_1$. In a previous paper it was suggested that as the responses of adenyl cyclase in rabbit intestine to cholera toxin and fluoride ion were overlapping, the mechanisms of stimulation by these two agents might be similar. However, the response to fluoride by adenyl cyclase under basal and toxin-stimulated conditions has been reported to be different in rabbit (26) and guinea pig small intestine. In guinea pig ileum, the enzyme activity in the presence of fluoride in cholera-treated tissue was greater than in control tissue. In rabbit ileum no difference was detected. When the latter studies were repeated using a larger group of rabbits, the activity of the enzyme was slightly but significantly higher with fluoride in the toxin-treated tissue compared with the control (Sharp & Witkum, unpublished). As the fluoride stimulation was maximal in these studies, the mechanisms of activation by fluoride and by cholera toxin are presumably different. However, as the increment of fluoride activation was significantly lower in the toxin-treated tissue than in the control, the effect of fluoride and cholera toxin is neither additive nor completely overlapping. Thus the mechanisms of stimulation of these two agents are different; because there is no proportionality between the increase of activity due to cholera toxin and the increase in fluoride response, the conclusion from previous studies that cholera toxin is not causing the synthesis of new enzyme is still valid. One further point which differentiates the stimulation by cholera toxin from the stimulation by fluoride can be seen in experiments using pyrophosphate. Pyrophosphate is known to inhibit the activation of adenyl cyclase by fluoride, while having little effect upon basal activity or stimulation by hormones. In studies with rabbit ileum pyrophosphate at a concentration of $5 \times 10^{-4} M$ inhibited the effect of fluoride by 60%, while inhibiting the activity of the toxin-stimulated enzyme by only 10%. It had no effect on basal enzyme activity (Hynie, Witkum & Sharp, unpublished).

Studies have also been performed on adenyl cyclase in mucosal epithelial cells of human jejunum, taken by biopsy within 24 hr of the onset of cholera (at a time when the average water loss was over 10 liters per day) and then again during convalescence when the diarrhea had stopped (29, 30). Under carefully standardized conditions adenyl cyclase activity during the diarrheal phase was more than double that during convalescence. Under these two conditions, no difference was observed in the characteristics of the enzyme other than an increase in the rate of reaction. This applies to the requirements for ATP, $Mg^{2+}$, and pH; the concentration dependence of the fluoride response; stability; loss of activity during homogenization; and the effects of $Ca^{2+}$, $Mn^{2+}$, and prostaglandins. Thus, the mechanism by which cholera toxin interacts with adenyl cyclase does not alter the properties of the enzyme, or at least those properties studied thus far.

Studies have been performed which localize the adenyl cyclase of intestinal cells to the basal and lateral membranes, and suggest that no adenyl cyclase is present in the brush borders (43). The membrane fractions were separated by homogenization and centrifugation techniques developed by Forstner et al (44) and Quigley & Gotterer (45). The brush borders were characterized by high alkaline phosphatase activity, low Na,K-ATPase, and by electron microscopic appearance. Basal and lateral cell membranes were characterized by low alkaline phosphatase activity, high Na,K-ATPase activity, and by electron microscopic appearance. In the latter, cholera toxin increased adenyl cyclase activity while decreasing the activity of Na,K-ATPase. The activities of alkaline phosphatase, leucine aminopeptidase, 5′-nucleotidase, glucose 6-phosphatase, and Mg-ATPase were unaffected by the toxin (43). Inhibition of mucosal Na,K-ATPase in cholera patients who had undergone intestinal biopsy was reported earlier by Hirschhorn & Rosenberg (46). The same enzyme inhibition was found in experiments in which a microsomal preparation of the intestinal mucosa was incubated in vitro with an unpurified *Vibrio cholerae* culture filtrate (47). Three conclusions may be drawn from these observations: 1. Adenyl cyclase, like Na,K-ATPase, seems to be absent in the brush borders of intestinal epithelial cells. 2. It follows that cholera toxin, present at the luminal surface of the intestinal epithelial cells during naturally and experimentally acquired cholera, does not affect adenyl cyclase in the brush border with which it is in immediate contact. Rather, it affects the enzyme in the lateral and basal plasma cell membranes. One reservation to this conclusion is that crypt cells, which do not have a brush border, may have adenyl cyclase in the luminal border because the isolation techniques used do not differentiate between smooth luminal membranes and the basal and lateral membranes. 3. Cholera toxin does not uniquely affect adenyl cyclase. It has in addition an effect upon Na,K-ATPase.

In trying to understand how the toxin acts, five salient features may be helpful. These are that (*a*) even brief exposure of the intestinal mucosa to cholera toxin results in a steady increase in fluid production; (*b*) there is a latent period of variable length before onset of the effect; (*c*) the effect of cholera toxin is persistent and is not abolished by washing the affected cell membranes; (*d*) the adenyl cyclase is not affected directly by a toxin interaction at the luminal surface of the cell; and (*e*) the effect is not specific for the one enzyme adenyl cyclase because Na, K-ATPase is also affected.

Numerous possibilities exist for the manner in which cholera toxin stimulates adenyl cyclase. For example, it has been suggested that cholera toxin could: 1. stimulate adenyl cyclase directly; 2. be converted to an activator of adenyl cyclase; 3. cause the release or synthesis of a stimulator of adenyl cyclase; or 4. remove a normal inhibitory factor.

1. For cholera toxin to stimulate adenyl cyclase directly, one has to account for the following properties of the toxin. It has a time course of effect which encompases a latent period of 15–60 min before a progressive increase of cyclase activity begins. This increase reaches peak values at 5 to 6 hr. Furthermore, brief exposure of the tissue to toxin (1–5 min) is sufficient to cause a full-fledged response. Thus, after toxin reached the cell surface, several hours would elapse before the toxin reached and fully activated the cyclase. This time course seems unlikely and the theory does not account for the effect of toxin on Na,K-ATPase.

2. Rapid uptake and gradual conversion to a stimulator of adenyl cyclase would be in accord with the time course of the effect of toxin and perhaps with the localization of immunologically reactive toxin in the apical (supranuclear) portion of the epithelial cells (48). Such an activator would presumably have a high affinity for adenyl cyclase because experiments designed to detect such an activator in the cytosol fraction of epithelial cells have been unsuccessful. If this is the case, then the activator could interact directly with adenyl cyclase in much the same way that a hormone receptor stimulates the enzyme. As a speculation along these lines, and in accord with the observation of Gorman & Bitensky (49) that the

effect of toxin overlaps that of epinephrine, the toxin product might mimic a fully activated β-adrenergic receptor.

3. Cholera toxin could enter the cells and then release or synthesize an activator of adenyl cyclase within the cell. Synthesis of a protein in the action of cholera toxin has been suggested by the finding that cycloheximide blocked fluid production in experimental cholera (50, 51). The full significance of this finding is not clear, however, because in the isolated fat cell increased lipolysis and increased adenyl cyclase activity were not blocked by cycloheximide (52). While such an action via synthesis would satisfy the time course of the effect of cholera toxin, the theory depends upon an indirect inhibitor study and requires further work to prove or disprove it.

The activators discussed most often as possible intermediates for the action of cholera toxin are the prostaglandins. Prostaglandins have been shown to stimulate adenyl cyclase (26, 27), and to cause fluid production in the intestine of composition similar to the fluid produced in cholera (21). A recent suggestion that prostaglandins are involved (53) and two reports that anti-inflammatory agents, which inhibit prostaglandin synthesis, will antagonize the effect of cholera toxin on fluid production in intestine (54, 55) have raised interest in the prostaglandins again. There are, however, several reasons for thinking that prostaglandins are not the mediators of the action of cholera toxin. For example, the stimulation of adenyl cyclase by cholera toxin is much greater than that which has been achieved by prostaglandins (26, 27). More important, after maximal stimulation by cholera toxin, adenyl cyclase is still responsive to prostaglandins (26, 27). The character of the stimulation is different and while the effect of cholera toxin to stimulate adenyl cyclase is relatively permanent, and persists even in well-washed membranes, the stimulatory effect of prostaglandins is not permanent and can be washed away. Finally, the effect of prostaglandins in the fat cell is not stimulatory but inhibitory (56). Thus the effects of the two agents can be dissociated. Cholera toxin stimulates adenyl cyclase in these cells and increases lipolysis, while prostaglandins inhibit lipolysis. Thus it seems unlikely that prostaglandins could be involved in the action of cholera toxin.

4. An action to remove inhibitory influences could also be a possibility. Adenyl cyclase can be stimulated in a variety of tissues by specific hormones (57, 58)—thus it is present in the membrane in a restrained condition. The nature of the control mechanism is not known, while the complications of its operation are only beginning to be unraveled. Recent studies have suggested that specific phospholipids are involved in the hormonal stimulation of adenyl cyclase (59–61). Thus one might speculate that as adenyl cyclase and Na,K-ATPase are both phospholipid-dependent enzymes, as both are affected by cholera toxin, and as phospholipase activity has been associated with *Vibrio cholera* (62), that phospholipase activity by the toxin could be involved. This brings up the interesting observations of Leitch & Glinsukon (63) that significant alterations in membrane chemistry can result from treatment with cholera toxin, including a loss of membrane phospholipids. Thus the toxin could be a phospholipase, a prophospho-

lipase which is converted to the active form by the intestinal cells, or an activator of cell phospholipase. The author favors an action of cholera toxin involving a membrane component, such as the lipids, over the previous suggestions because it satisfies the five salient features of the action of cholera toxin mentioned previously. Thus, the rapid uptake would be followed by a latent period in which a change in membrane composition would begin, progressive change would cause the effect to be persistent, a direct interaction with adenyl cyclase at the luminal border is not required, and the toxin could affect other membrane-bound enzymes in addition to adenyl cyclase. If this theory is correct, then cholera toxin should be a useful tool for investigation of the mechanism of control of membrane-bound enzymes.

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7057

# DRUG METABOLISM IN THE NEWBORN[1]

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## INTRODUCTION

The developing fetus and newborn have been shown repeatedly to be more sensitive than the adult to various pharmacological agents. The nature, duration, and intensity of drug action is dependent upon many different factors such as volume of distribution, protein binding, metabolism, excretion, and receptor sensitivity, as well as intrinsic properties of the drug itself. The newborn infant differs from the adult in many ways with regard to the above-mentioned functions, and generally they are less developed than in the adult. This review will be limited to only one of these factors, namely the capacity to metabolize drugs. Emphasis will be placed on the neonatal period, but recent investigations of drug metabolism in the fetus warrant inclusion of selected fetal studies.

The purpose of drug metabolism is to render foreign compounds polar and more water soluble. As a result, excretion into the urine is facilitated while passage across tissue membranes, such as the placenta, is decreased. This transformation is generally achieved through different conjugation processes such as glucuronidation, sulfation and glycine coupling with or without prior oxidation, reduction, or hydrolysis. The majority of these reactions are catalyzed by enzymes located in the microsomes of the liver. The physiological icterus, which occurs in approximately two thirds of all newborn infants, has long been suspected to be due, at least in part, to hepatic immaturity. It is, however, only during the last two decades that increasing interest has been directed towards the capacity of the developing liver to metabolize drugs.

This presentation will first discuss information gathered through animal experiments and conclude with clinical investigations in the neonate. Special attention will be given to the problem of enzyme induction and its therapeutic relevance.

## ANIMAL STUDIES

With the knowledge that a majority of newborn infants develop unconjugated hyperbilirubinemia, glucuronidation was one of the first systems to be studied.

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Low or negligible values were found both in animals and humans with a variety of aglycone acceptors (1, 2). Further studies indicated that the enzymes in the last two steps of glucuronide formation, glucuronyl transferase and UDPG dehydrogenase, were deficient in activity when tested in vitro. This low capacity was also found for different oxidation reactions (3, 4). These first studies in mice, rabbits, and guinea pigs were later followed up and confirmed in additional animal species (5–7). In vitro studies have later been supplemented with in vivo measurements of drug effects such as sleeping time and determination of plasma concentrations as an indicator of in vivo metabolism (8). During the postnatal and suckling period, drug metabolic capability increases more or less rapidly and reaches maximal activity at varied ages according to the pathways under investigation. Some pathways, for example glucuronyl transferase and sulfation, reach even higher values during this period than later in life. However, exceptions to the general concept that all newborn animals have low drug metabolizing capacity can be found for reduction (7) and sulfation (9). Both reactions are well developed at birth with adult levels of in vitro activity.

The human fetus differs from the experimental animal in that drug metabolizing activity can be found in vitro as early as the second trimester, as will be discussed later. This, of course, generated some doubts as to the validity of extrapolation of animal data to man. In addition to describing the developmental biochemistry and pharmacology of a specific animal, the experimental design can be used to answer certain questions, several of which will be discussed here.

One of the major questions which immediately arises concerns what initiates drug microsomal enzymatic activity at birth. Several explanations have been offered and intense work is under way on this subject. A possible explanation is the disappearance of an inhibiting substance in the pregnant and fetal animal (4, 10). The existence in rats of a lower drug metabolism during late pregnancy was taken as indirect evidence for this theory (10). Rats weaned early and therefore removed from the influence of the mother had a higher 4-methylcoumarin hydroxylase activity then their unweaned counterparts. However, prolongation of weaning did not effect the development of aminopyrine demethylation (6). We have found similar results with hexobarbital oxidation in the mouse, but in contrast to Henderson's findings, prolongation of weaning did affect the activity of this enzyme system (11).

It seemed appropriate to relate these observations to the profound hormonal changes that take place at birth and at weaning. Attention has been primarily focused on progesterone and growth hormone. Progesterone and some of its metabolites have been shown to inhibit liver mixed-function oxidase activity, though other factors, such as decreased receptor threshold and increased permeability, have been shown to be important for the newborn rat's increased sensitivity to pregnenolone (12, 13). Recently, a greater proportion of progesterone was shown to be reduced by the maternal liver during pregnancy in rats (14). The treatment of newborn rats with these reduced progesterone metabolites resulted in decreased hydroxylase activity. In addition, treatment of weaned rats with the same metabolites prevented the usual increase in activity of hydroxylase metabolism associated with separation from the mother. However, these inhibitory substances should be present in maternal or fetal plasma and no in vitro inhibition of hexobarbital or aniline metabolism was observed after addition of various amounts of plasma from 10 day old suckling rats or lactating females (15).

Growth hormone has been shown to impair drug metabolism in both immature and adult rats, suggesting that it should play a regulatory role in hepatic microsomal drug metabolizing enzyme activity (16). Implantation of a growth hormone secreting pituitary tumor into young rats or administration of growth hormone to immature rats both impaired drug metabolism and prevented its age-related development. In adult rats, hexobarbital metabolism could be decreased to the level normally seen in 20 day old rats by the same treatment.

Absence of necessary cofactors in the fetal animal is another possible explanation of the low metabolizing activity. No experimental data exist to either support or negate this view. In adult animals, drug metabolizing activity has been correlated with liver glycogen content (17). Liver glycogen is known to accumulate during the later half of pregnancy in all mammalian species (18). Studies in pigs showed no correlation between liver glycogen content and the development of different enzymes concerned with drug metabolism (7).

The lack of ability to synthesize enzyme protein in the fetal liver has been another consideration. Inhibition by actinomycin or puromycin of the expected increase in glucose 6-phosphate activity and partially of NADPH cytochrome *c* reductase activity was taken as an indication that a new and rapid synthesis of these enzymes actually took place postnatally (19). An unchanged ATPase level was further proof that these substances had only inhibited the RNA and subsequent protein synthesis and had not caused cellular damage.

Another interesting area has been the comparison between fetal, infant, and adult enzyme systems and the possibility of differences in the structure and nature of the enzyme protein. In studying the synthesis of L-ascorbic acid in rats, it was shown that microsomes of fetal rats are different in that they sediment at a much lower centrifugal speed (20). The differences in the pattern of inhibition of varying salt concentrations on hepatic phenolsulfo-transferase activity in 1 and 28 day old mice suggested that the protein structure is not the same (21). In contrast, no differences in PCMB sensitivity were found between 5 day old and adult rat microsomes, and this was considered to be an indication that NADPH reductase is similar at both stages (19).

Most work concerning the development of drug metabolizing activity has been carried out in normal animals, but it is known that environmental factors influence the activity. Enzyme induction will be discussed under a separate heading. Another factor that may influence the capacity to metabolize drugs is the animal's nutritional status (22, 23). Total starvation usually results in a diminished activity, while specific deficiencies may have opposite effects. Very little is known regarding these effects in the developing animal. By feeding a lactating rat a diet supplemented with various fats it was possible to change the fatty acids of the phospholipids in the microsomes of the suckling animals (19), but no changes in the predicted development of enzymic activities were noted. Malnutrition in suckling animals produced by increasing the number of animals in the litter from 10 to 18 resulted in an enhanced in vitro capacity for conjugation and aminopyrine metabolism, while reductive pathways were decreased (24). When drug metabolizing activity was assayed in vivo by hexobarbital sleeping time, a significant prolongation in the malnourished animal was found, though both experimental and control animals woke up at the same brain and blood levels of the drug (25).

Besides environmental factors, the genetic makeup influences drug enzyme activity. These factors are already in operation at birth as shown by differences in hexobarbital sleeping time between different inbred strains of mice (21).

Lastly, it might be speculated that the diminished drug metabolizing activity in the newborn animal might not always be a disadvantage. For example, phalloidin is lethal when injected into adult but not newborn rats (26). This effect may be caused by a toxic metabolite of the drug which is formed only in the mature liver.

## HUMAN FETUS

As mentioned earlier, drug metabolizing activity for several pathways is almost absent or very low in animal fetuses regardless of developmental maturity at birth. The human fetus, on the other hand, differs from experimental animals and acquires this ability as early as the third month of pregnancy. So far, drug metabolism in the human fetus has only been studied in vitro with tissues, mainly liver, obtained through legal abortions. Sedimentation properties similar to those in rat fetal tissue (20) were found using glucose 6-phosphatase as a microsomal marker. The greatest proportion was found in the low speed pellets which also correlated with the enzyme activity of NADPH cytochrome *c* reductase and P450 content (27).

Synthetic reactions including conjugation with glucuronic acid and ester sulfation were the first to be studied. As in animal fetuses, low values were found for glucuronyl transferase and UDPG dehydrogenase in three month old human fetuses (2). In contrast to this, ester sulfates could be formed in vitro with phenols and various endogenous substrates in early pregnancy (28).

Oxidative capacity was first studied with *p*-nitroanisol and *p*-nitroaniline as substrates for O- and N-demethylation, and no activity could be found in 3–10 month old fetuses (29). Similar results were obtained for 3,4-benzpyrine and aminopyrine metabolism whereas the liver was very capable of oxidizing endogenous substances such as testosterone and laurate (30). However, evidence has now been accumulated that oxidation and reduction can take place in vitro for such substrates as chlorpromazine, meperidine, and *p*-nitrobenzoic acid (31). The activities were generally low, but a trend could be seen towards an increasing activity with increasing fetal weight. Kinetic constants for chlorpromazine oxidation and *p*-nitrobenzoic acid reduction were of the same magnitude as in adult animals and this was interpreted as indicating a similarity between the enzymes. It was also suggested that earlier failure to find enzyme activity was due to the lack of methods sensitive enough to detect small amounts of metabolites. Recently, aniline hydroxylation and demethylation of ethylmorphine were found to be 30–40% of adult activity (32). Alcohol dehydrogenase could be detected in fetal liver at 3–4% of adult activity (33). When the fetal and adult enzymes were compared, a slight change in pH optimum and different $K_m$ values were found. Electrophoretic patterns showed four bands for the adult, two for the full-term infant, and one for the fetus (34).

A prerequisite for all types of microsomal drug oxidation is the presence of cytochrome P450. Drug oxidation is thought to involve binding of a substrate to the oxidized form of cytochrome P450 and subsequent reduction of this complex by a flavoprotein cytochrome P450 reductase. It was therefore of great interest to show that these components could be found in human fetuses in the second trimester and in amounts of the same magnitude as in adult liver biopsies when calculated per gram of liver tissue (27, 30, 35). All substrates to be oxidized bind to cytochrome P450 before final combination with oxygen and this is reflected in spectral changes. When spectral changes were examined, human fetuses were found to differ from adult human or rat. For example, the fetus bound testosterone with a type I spectral change while adult human tissue exhibited a reverse type I binding. Aminopyrine is bound to fetal P450 in a type II way, while the adult binds it with a type I spectral change. This has been interpreted as a possible indication that different fetal and adult P450 forms might exist.

Lastly, even if data exist for the presence of the metabolizing activity in fetal liver, as well as in other fetal tissues such as kidney and intestines (31), nothing is known about in vivo capability or function. While studying aniline metabolism, it was noticed that methemoglobin was simultaneously formed (36). This was suggested to be a potentially harmful effect of fetal metabolism since the fetus is known to have a low capacity to reduce methemoglobin.

## HUMAN NEWBORN AND INFANT

In contrast to studies in the fetus, only one in vitro study has been reported with respect to newborn infants (37). The remaining investigations of drug metabolism in this age in the human have been carried out in vivo. NADPH cytochrome *c* reductase activity and cytochrome $b_5$ content were studied postmortem in the liver in one premature and one full-term newborn. The properties of NADPH cytochrome *c* reductase were shown to be similar to those of adult rats.

Drugs can reach the newborn by direct administration or through several different indirect routes such as the placenta or breast milk, both following maternal intake and treatment. So far, however, most studies on drug metabolism in the human infant have been performed following drug administration for therapy of the infant. Regardless of the route and source of administration, drug metabolism in vivo is usually studied measuring plasma half-lives and/or identification and quantitation of metabolites excreted in the urine. Many of these investigations have been limited due to the minimal amount of blood that can be drawn from a small infant. However, sensitive analytic techniques are being developed at the present time such as mass fragmentography (combination of mass spectroscopy with gas chromatography), which will permit study of the newborn with ultra micro sample size.

Relatively few studies have been performed after transplacental administration of drugs. Of the few drugs studied so far, all have a prolonged elimination rate into the urine. Following administration of therapeutic doses of chlorpromazine, pethidine, and promazine to the mother during labor, the urine of the neonates was analyzed for the presence of metabolites and/or parent drug (38). It was found that the urine contained other conjugated metabolites than the ones excreted in adults. This was taken as an indication of a different metabolism in the newborn. A prolonged half-life of nortryptiline and evidence that the newborn possesses the capacity to metabolize this drug was found when a pregnant woman took an overdose of the drug one day prior to delivery (39). Newborns of mothers taking diphenylhydantoin were shown to excrete the drug in a significant amount only on the third day of life. This gave a half-life of 60 hr compared to 12 hr in the adult (40). Ethanol elimination was twice as long in the newborn as in the mother following maternal administration (and transplacental passage) 3 hr prior to delivery (41). No adverse effects were seen in either the infant or the mother.

Most drugs studied after direct administration to the human infant have also pointed to a decreased drug metabolizing capacity, shown mostly by a slow elimination rate. In contrast to this, it was surprising to find lower concentrations of diphenylhydantoin in plasma of neonates than in adults treated with the same dose(41a). DPH is hydroxylated and conjugated in the liver before being excreted. However, no assessment of the urinary metabolites was performed. With regard to digoxin, for example, the finding of a greater proportion of it in its unchanged form in the urine was taken as an indirect evidence for diminished metabolizing capacity in the newborn (42).

The best-known example of hepatic immaturity and decreased ability to form conjugates is probably the physiological jaundice that develops in more than two thirds of all newborn infants. This was analyzed in greater detail by giving intravenous bilirubin as a test substance (43). It was shown that the plasma half-life was significantly prolonged up to the second month of life as compared to older children. Conjugating capacity was further studied with sulfobromophthalein and *p*-aminobenzoic acid. Sulfobromophthalein is conjugated with different amino acids and glutathione, and *p*-aminobenzoic acid with glucuronic acid and glycine before being excreted. The plasma half-life of sulfobromophthalein was studied in 121 healthy, mature, and premature newborn infants and children (44). Half-lives were longer in premature infants than in mature infants of the same age, though by the postnatal age of 4 months the half-lives became the same in both groups. It was then concluded that exogenous factors were more important than endogenous for the maturation of the metabolizing system. The underlying mechanism for the increased retention of sulfobromophthalein in the newborn was suggested to be not only hepatic immaturity, but also insufficient secretion into the bile (45). *p*-Aminobenzoic acid, which is coupled to glucuronic acid and glycine, and *p*-aminophenol (the oxidized metabolite from acetanilid), which is glucuronidated before excretion, were both eliminated more slowly in newborn and premature infants (46, 47). In addition to the delayed conjugation with glycine for *p*-aminobenzoic acid, quantitatively different patterns of urinary metabolites were found. Acetylation at the amino group was more prominent in younger children. In contrast, the ability to acetylate sulfadiazine was found to be decreased in young children (48).

The decreased ability of small infants to metabolize drugs may become hazardous if not taken into account when administering such drugs as chloramphenicol and nalidixic acid, which are both glucuronidated (49, 50). Following prophylactic use (especially in premature infants) of chloramphenicol, an increased incidence of deaths in cardiovascular collapse, the so-called Grey syndrome, was seen. When pharmacokinetic studies were conducted in further detail at this age, unusually high plasma concentrations of the drug were found. The toxic reaction is, however, not only due to hepatic immaturity, but to a combination of unfavorable factors including kidney immaturity.

Oxidative capacity has been studied using several different drugs and generally has been shown to be deficient as well. When acetanilid was given to 10 newborn infants, the peak concentration of the oxidative product *p*-aminophenol appeared later than in older children (46). Tolbutamide was administered orally and intravenously to 10 normal full-term babies and a prolonged plasma retention was found during the first two days of life (51). The plasma disappearance showed an inverse correlation with the appearance of the oxidized metabolite carboxytolbutamide in the urine. Further evidence for a decrease in oxidative ability was gathered when amidopyrine half-lives were measured on the first and eighth days of life in 15 normal full-term infants (52). A depressed capability for reductive metabolism of cortisol was suggested by the increased excretion of both exogenous and endogenous cortisol in its free form (53).

So far, all the studies discussed have been performed by the authors themselves. However, the possibility of further studying or reevaluating already published studies employing techniques of another discipline should not be forgotten. This approach has recently been applied to further elucidate the mechanism behind the longer duration of succinylcholine action in some newborn infants (54). For 2 of the 9 infants, a slower elimination of the drug was clearly shown by means of pharmacokinetic analysis of the data.

## INDUCTION OF DRUG METABOLIZING ACTIVITY

*Animal studies.*—A wide variety of environmental factors, such as drugs, have long been known to enhance liver microsomal drug metabolism in the adult experimental animal. It thus seemed logical to investigate whether this effect could be utilized to modify low or absent enzymatic activity in newborn animals. Both oxidative and reductive pathways were increased following pretreatment of pregnant rabbits with phenobarbital at term or of the newborn rabbit itself (55). This was later confirmed in other animal species such as the rat where an increase in in vitro metabolism of phenobarbital was correlated with an enhanced plasma disappearance of this drug (56). Besides different barbituric acid derivatives, so far only a few other substances have been used as inducing agents in the perinatal period (57). Another interesting phenomenon is that induction can only take place in the later part of gestation (55). Exposure of infants to phenobarbital from breast milk is sufficient to induce an increase in metabolism (58). The increased metabolism induced by treatment of the mother one week pre- and one week postnatally can still be observed at 21 to 28 days of age in the young rats, while in adult animals induction lasted only 5 to 7 days following discontinuation of treatment (59).

The mechanism behind the increase in drug metabolism following treatment with phenobarbital is not fully clarified. Phenobarbital has been shown to increase microsomal protein content. The amino acid antagonist ethionine injected into 24 hr old rabbits prevented the increase in hepatic drug metabolism normally caused by phenobarbital (55). Phenobarbital also resulted in increased hepatic uptake and enhanced excretion rate into the bile of different compounds as well as an increase in biliary flow (60). Concerning the increased hepatic uptake, two cytoplasmic fractions designated Y and Z involved in the binding of bilirubin and BSP have recently been demonstrated (61). Phenobarbital administration enhanced uptake of BSP and simultaneously increased the amount of Y protein (62). In fetal and neonatal rat tissue using gel absorption techniques, it was shown that binding capacity of hepatic carrier protein for bilirubin and BSP was low, but increased rapidly and reached adult values at 30 days of age (63). In newborn monkeys, the intracellular concentration of Y protein in the liver tissue was low, and the postnatal increase in Y protein corresponded to the change in plasma clearance of BSP and serum bilirubin (64).

*Studies in man.*—The knowledge of enzyme induction was first applied to children with unconjugated hyperbilirubinemia (65, 66). Phenobarbital treatment caused a rapid decrease of bilirubin in a child with familial unconjugated hyperbilirubinemia and this was interpreted as an indication of induction of glucuronidation. Inferentially, this was demonstrated by simultaneously administering a low dose of salicylamide and finding an increase in output of its glucuronide in the urine (65).

Following this observation, interest was directed towards the same treatment of the normal physiological jaundice of the newborn. Retrospectively, children of epileptic mothers treated with phenobarbital were shown generally to have lower serum bilirubin concentrations (67). In several studies, phenobarbital has been given both to the mother and the newborn and significantly lower bilirubin concentrations were found when compared to untreated control groups. These data have recently been reviewed. The same indirect method described earlier, estimation of the excretion of salicylamid glucuronides, was applied to these infants to demonstrate that in fact a stimulation of glucuronidation follows phenobarbital treatment (68). As mentioned earlier, the mechanism underlying the decreased plasma concentrations is probably complex. An increase in sulfobromophthalein clearance was observed 24 hr following phenobarbitone treatment of jaundiced infants (69). The shape of the disappearance curve suggested an enhanced hepatic uptake as well as excretion of the dye.

Besides bilirubin conjugation, the oxidative pathway of amidopyrine has been studied after phenobarbital treatment in newborn infants (52). The treated group of infants showed a significant decline in serum bilirubin concentrations as expected, but no further increase in amidopyrine elimination could be seen except for that accounted for by the maturation process itself.

In addition to induction with barbituric acid derivatives, trials have been made with a few other drugs such as phenylbutazone, diethylnicotineamide, and ethanol. Although the effects were not as pronounced as those after phenobarbital, a decrease in bilirubin level was also found following phenylbutazone treatment (52). Diethylnicotineamide was studied following administration to one of ten twin sets twice daily from the second to the ninth day of life and a significantly lower bilirubin value in serum was found 96 hr after starting treatment (70). Ethanol infusion given to pregnant women from 3 to 96 hr prior to delivery resulted in a lower mean maximum serum level of bilirubin as compared with untreated controls (71). In contrast to animal studies, induction seems to take place even during early pregnancy, at least when this capacity is assayed in vitro (72).

In this connection, the possibility of inhibiting drug metabolism should also be mentioned. In a few infants of nursing mothers, persistent jaundice can be found which disappears when the infant is weaned to bottle feeding (73). When breast milk from these mothers was added to rabbit liver homogenates, an inhibition of *o*-aminophenol glucuronidation was found and the inhibiting substance was later shown to be pregnanediol. However, no correlation was found between serum bilirubin levels and the inhibitory effect in vitro of corresponding milk samples (74).

## CONCLUSION

In this review article we have tried to summarize what is known from animal and human data about drug metabolism in the newborn period. We have pointed out how animal experiments can serve to answer certain basic questions which later can be used in treatment of human infants. Phenobarbital therapy of neonatal hyperbilirubinemia is an example of this concept. Caution has been urged in extrapolating animal data to the immature organism. The recent demonstrations of the presence of the components of the oxidative electron transport chain, as well as oxidative metabolism in midpregnancy, stands in marked contrast to their absence in several animal species during fetal life.

So far, most studies have been conducted in normal animals and in normal full-term or premature human infants. It can be speculated that various disease states may influence drug metabolism in a way which would differ from the expected pattern and this may be of great significance in adjusting drug dosage in the sick newborn infant. Studies in this regard are sorely needed if the tragedies of the past are not to be repeated.

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# THE LESCH-NYHAN SYNDROME[1]

7058

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The Lesch-Nyhan syndrome is a heritable disorder of purine metabolism that was definitively described in 1964 (1). The responsible gene is on the X chromosome, and the disease is transmitted as an autosomal recessive characteristic. The molecular site of the defect and the primary product of the gene is the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) (2). The most prominent metabolic consequence of this defect is an enormous oversynthesis of purine de novo (1, 3), which leads to markedly increased amounts of uric acid in body fluids.

## CLINICAL FEATURES

The cardinal clinical characteristics of the syndrome are mental retardation, spastic cerebral palsy, choreoathetosis, and self-mutilative biting behavior (1, 4, 6–8), associated with hyperuricemia, urinary tract stone disease, nephropathy, gouty arthritis, and tophi (4, 5). The Lesch-Nyhan syndrome occurs exclusively in males who appear normal at birth and usually develop normally for the first six to eight months. Crystalluria, hematuria, or renal tract stone disease may develop during these early months of life, but in most patients the neurological examination is negative.

The onset of cerebral manifestations is with athetosis. Infants who have been sitting and holding their heads up will begin to lose these abilities. Initially they may be hypotonic or hypertonic, but deep tendon reflexes are increased. Later they are all markedly hypertonic. In the established patient the motor defect is of such severity that he can neither stand nor sit unassisted. Patients may learn to sit in a chair only if they are securely fastened about the chest. They much prefer being upright and are best managed by tying them securely in a chair with arm and leg rests. All patients described have been mentally retarded. However, their motor defect is of much greater severity than their defect in intelligence, and this makes intelligence difficult to test in childhood. Most of our patients have had IQs of 50 or less. However one boy has a borderline IQ and another appears to be of normal intelligence. On the other hand, none of the patients has been successfully toilet trained. None has walked alone.

Some of the patients have been quite irritable, inconsolably colicky babies.

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[1] The survey of the literature on which this review is based was completed in August of 1972. This review was aided by a grant from the Health Services and Mental Health Administration, No. 274, U. S. Department of Health, Education, and Welfare.

However, this could relate to crystals and stones in the urinary tract as well as to the nervous system, but in those we have spent some time with, the irritability and screaming appeared to be central in origin. Alterations in muscle tone occur early (8–11) and ultimately lead to marked muscular hypertonicity. This has in some progressed to contractures and to dislocation of the hips. Scissoring of the lower extremities is prominent. Deep tendon reflexes have uniformly been increased. Some patients have had Babinski signs. Many have had ankle clonus.

Involuntary movements of both choreic and athetoid type have been prominent. In fact, choreoathetoid cerebral palsy may be the most consistent feature of the syndrome. Choreoathetosis is increased with tension or excitement. The degree of tone and involuntary movement is such that the patients sometimes resemble those with dystonia. They are particularly characterized by sudden opisthotonic or extensor spasms of the trunk. As the patient gets older these spasms begin to appear to be at least semivoluntary. It can be hazardous to be directly behind such a patient when his head is suddenly thrown back in extreme extension. It can also be hazardous for the patient if he is on a hard surface, for he may lacerate or otherwise injure his head.

The speech is characterized by athetoid dysarthria. As the patient gets older this problem in communication becomes particularly frustrating, and of course frustration and anger only increase the dysarthria. On the other hand, those who work day to day with these patients usually learn to understand their language. Athetoid dysphagia is another problem, and this can be life-threatening. These patients have so much difficulty swallowing that they are very difficult to feed. Furthermore, they all vomit frequently. In a busy, crowded state hospital, such a patient may die of inanition. Most of them are markedly underweight and many are quite short. In addition, they aspirate frequently, and pneumonia is common. Convulsions are not a regular feature of the syndrome, but they have been observed in a number of patients.

Aggressive, self-mutilative behavior is probably the most striking aspect of the syndrome (1, 8, 12, 13). Self-mutilation may begin as early as the eruption of teeth. It usually begins at least shortly thereafter. Most patients bite both their lips and fingers destructively. Every patient we have seen has bitten his lip destructively, unless the primary teeth have been removed very early. In most patients the hallmark of the syndrome is loss of tissue about the lips. Partial amputations of the fingers are common.

Self-mutilation is not uncommon in populations of retarded children. However, it very seldom takes the form seen in this syndrome. With a few exceptions, patients can be diagnosed as having this syndrome at a glance. Most retarded children who injure themselves do not produce loss of tissue. More often they pick, bite, or hit at some part of their body, or beat it against a hard surface. The resultant lesion is usually one of hypertrophy, callus, or swelling, rather than loss of tissue.

Anesthesias come to mind in the differential diagnosis of self-mutilation (14). Patients with sensory neuropathy, congenital indifference to pain, or dysautonomia may all have signs of self-mutilation. They all look quite different from hyperuricemic children. The child with a congenital anesthesia generally looks like a pugilist. He may have unrecognized fractures and may burn or lacerate his fingers without his knowledge. In children with the Lesch-Nyhan syndrome, sensation is intact. They scream in pain while they bite themselves, and they are really happy only when securely protected from themselves by physical restraint. Many of these children scream all night until their parents or guardians are taught how to restrain them securely in bed.

In contrast it should be said that these are unusually engaging children when they are restrained. They have a good sense of humor and smile and laugh easily. They appear to most of those close to them, both professionals and relatives, to have a higher intelligence than their tests scores and motor limitations would indicate. They are often special favorites of ward personnel. Patients with the syndrome tend to look quite a bit alike. This, of course, is true of athetoid children in general, but it is more true of these patients. They are reminiscent of the principal character in the ballet "Petroushka," with their tragicomic air, exaggerated athetoid posturing, and mitten-like protective coverings on their hands.

When protective coverings or restraints are removed their personality changes immediately. They appear terrified. They scream and yet compulsively they bite at themselves. Mutilation could not come from biting alone. These patients are generally destructive. We have seen children who have lacerated themselves in braces or caught themselves in the spokes of wheelchairs. They may find hot water faucets and burn themselves. One boy burned himself in a dry ice acetone bath left unattended by an investigator in a clinical research unit. As they get older they learn to call for help.

Their behavior is not directed solely against themselves. This is compulsive aggressive activity. They are limited in their aggressions against others by their motor defect, but they will bite others if given the opportunity. Physicians and nurses working with them are often kicked or hit, and broken eyeglasses are common around these patients. They often laugh uproariously when successful in hitting someone. On the other hand, they are just as often remorseful about having produced injury. As they get older and learn speech they become verbally aggressive as well. Pinching and grabbing at genitals and related areas may develop. Four-letter Anglo-Saxon expressions are common.

The behavior of these patients is a striking and provocative element in the syndrome. To our knowledge this is the first instance in which a stereotyped pattern of human behavior has been associated with a distinct biochemical abnormality. Understanding of mechanism could contribute to an understanding of behavior and its biochemical basis.

In the first few years after the description of the syndrome, a large number of case reports appeared. A scholarly summary of these early reports has been assembled (15). It has since become apparent that the syndrome is relatively common. Individual cases are no longer reported unless they illustrate some point made by an author reporting the results of his research.

A number of clinical manifestations are related directly to the accumulation of uric acid in the body fluids. Patients with this syndrome have hyperuricemia from the first days of life. As a consequence they are subject to all of the clinical features found in gout. Acute attacks of arthritis develop only after a number of years of hyperuricemia. Three of our older patients have had acute arthritis, and in one we were able to observe an effective antiinflammatory response to colchicine. An involved cousin of one of our patients was said to have died at 21 years and to have had repeated episodes of arthritis in his last year.

Hematuria and crystalluria are common. A very early finding may be the appearance of masses of orange crystalline material in the diapers. A number of patients have had urinary tract stones (3, 5). Infantile colic and recurrent abdominal pains in older children may relate to the presence of large amounts of insoluble material in the urine. Urinary tract infections have been common only in those with stones. Marie, Royer & Rappaport (16, 17) have emphasized the occurrence of a renal concentration defect, associated with polyuria resistant to pitressin. While I have some doubt that their 18-year-old patient had the Lesch-Nyhan syndrome, he certainly had a related disorder, and we have seen polyuria, polydipsia, and failure to concentrate in typical examples of the syndrome. These patients may have real troubles satisfying their thirst in a large, busy institution. This tubular defect is clearly related to uric acid. Early in its course, it may respond to alkalinization or allopurinol. Later permanent renal changes occur. They affect the glomeruli as well. Urate nephropathy may lead to renal failure, and this has probably been the most common cause of early death.

Tophi also develop; we have seen them in three patients. Biopsy of one revealed the typical gross and microscopic appearance of sodium urate deposits in tissues. Furthermore, the tophaceous material was a substrate for uricase. One patient had a tophus on his ear as large as a golf ball. Another has had tophi which have broken down and drained solid white urate.

Vomiting is a persistent problem in these patients. They may present in the first days of life with severe vomiting (18). They nearly all grow poorly and have shortness of stature and delayed bone age or skeletal maturation. The weight tends to be proportionately lower than the height in relation to standards for the patient's age. Endocrine assessments of growth and maturation are normal.

Anemia is observed with some frequency. Sometimes it is macrocytic and megaloblastic (19, 20). The anemia often regresses spontaneously. We have observed an associated eosinophilia in one patient, as has Michel (15).

Autopsies have been carried out in four patients, three of which have been reported. In one (21) some cerebral pathology was observed. There were granules which took the de Galantha stain for urate. In addition there was axonal swelling and demyelination of the white matter. Changes like this have been seen in other patients with uremia and are generally thought not to be relevant to the cerebral defects in the Lesch-Nyhan syndrome. The patients described by Crussi (22) did not have these lesions in the brain, nor did our second patient. The myelin displayed remarkable integrity. There were no microscopically discernible lesions in the basal ganglia. Micrencephaly has been observed, but not regularly. This might be consistent with the general failure of growth and development seen in many of these patients, which may well be nutritional.

## METABOLIC FEATURES

In most patients the first evidence of metabolic abnormality is the elevated concentration of uric acid in the blood. In children with the Lesch-Nyhan syndrome, uric acid concentrations have usually approximated 10 mg/100 ml. Normal concentrations have been observed in some patients occasionally, and in some regularly.

The excretion of uric acid in the urine is always elevated. Children with this syndrome excrete three to four times as much uric acid as do control children of comparable size. The 24 hr output of over 600 mg per day has been seen regularly in patients with the Lesch-Nyhan syndrome. Such a patient weighing 15 kg with an excretion this high is obviously a four to five times greater hyperexcretor than a 70 kg adult. We have therefore expressed uric acid excretion in mg/kg. Patients generally excrete from 40 to 69 mg/kg. Another way of expressing urinary excretion is in terms of the excretion of creatinine. These patients usually excrete 3 to 4 mg uric acid per mg creatinine, while in control individuals the value is generally less than 1.

The overall metabolism of purines has been studied by determining the rate at which uric acid is synthesized from small molecules such as glycine. This has been done by administering glycine isotopically labeled with $^{14}$C (1, 3). The uric acid is isolated from the urine, purified, and its specific radioactivity determined. In patients there is a rapid peak of specific activity in the first 24 hr. At the peak it is easy to distinguish patients from controls. In such studies in adults, it has not been possible to distinguish gouty from control populations. Therefore, it has become conventional to express the data as the cumulative percentage of administered glycine converted to uric acid. Adults with overproduction gout convert about twice as much glycine to uric acid as do controls. In children with the Lesch-Nyhan syndrome a mean of $>2\%$ of the glycine administered was recovered in uric acid (1). When compared with control children a difference of 20 times was obtained (4). These are the highest rates of overproduction of purine reported. These data indicate that the biochemical abnormality is as striking a deviation from normality as the clinical abnormality.

Uric acid pool size and its rate of turnover have been studied using uric acid-$^{14}$C (1, 18). We found a mean pool size of 43 mg/kg, similar to values reported in adults with nontophaceous gout. In a somewhat younger patient, 15 months of age, Jeune and colleagues (18) found a pool size of 70 mg/kg. Our turnover rates were 1.6 and 2.1 pools per day, and Jeune's was 3 pools per day. These are extremely high rates of turnover for uric acid, all of them higher than any previously reported in adults. Our children had net turnovers of over 1 g uric acid per day.

It is also possible to study the rate of de novo synthesis of purine in vitro using fibroblasts grown in cell culture (2). In the presence of azaserine the de novo pathway is blocked at the step in which the amide nitrogen of glutamine is transferred to formyl glycinamide ribotide (FGAR) to yield formyl glycinamidine ribotide. Isotope provided as $^{14}$C-formate that would normally end up in purine is trapped in FGAR. Thus the conversion of formate to FGAR is a measure of the de novo synthesis of purine. Patients with the Lesch-Nyhan syndrome clearly differ from controls in this assay (2).

It has been thought that the dramatic overproduction of purine de novo observed in this syndrome represents a failure of feedback control. This is a logical hypothesis. The first committed step of the purine synthetic pathway, catalyzed by phosphoribosyl pyrophosphate amidotransferase, is subject to feedback inhibition by the mononucleotides of adenine and guanine. If overproduction were simply a manifestation of failure of inhibition, one might have thought that these very rapid rates of purine synthesis were maximal. We tested this hypothesis using 2-ethylamino-1,3,4-thiadiazole. This uricogenic agent markedly increases the rate of purine synthesis in normal individuals. In the patients this compound increased the rates of purine synthesis even more impressively (23).

In the presence of a general overproduction of purine it is of some interest to consider compounds other than uric acid. Among the oxypurines these patients excrete xanthine in about the quantities found in normal urine. However, the amounts of hypoxanthine are markedly increased (24, 25). In most individuals the molar ratio of hypoxanthine to xanthine is less than one. In these patients the ratio is considerably greater than one. These differences are more dramatic when the patient is treated with allopurinol, which inhibits xanthine oxidase, decreasing urate excretion and making the other oxypurines the products of the overproduction. The hypoxanthine to xanthine ratio decreases with small doses of allopurinol, but as the degree of the block is increased, more and more purine ends up in hypoxanthine. In controls treated with allopurinol most of the oxypurine excreted is xanthine. These observations indicate that in normal individuals most urinary urate is formed from xanthine which does not come from hypoxanthine.

This finding is consistent with the occurrence of xanthinuria in patients with congenital absence of xanthine oxidase, abnormalities reflected in the central nervous system. Uric acid is not formed in the brain. These other oxypurines are the endproducts of purine metabolism there. In the cerebrospinal fluid (26) xanthine concentrations are identical to those of controls. The concentrations of hypoxanthine in patients with the Lesch-Nyhan syndrome were greater than in controls by a factor of four. Treatment with allopurinol increased the concentration of oxypurines in the cerebrospinal fluid of the patients by about three times.

We have undertaken a detailed analysis of the purines of the urine (27) to assess the possibility of other products of overproduction in these patients. The analysis was carried out on one patient and one control. In addition to the oxypurines, the patient differed from the control in the excretion of amino imidazole carboxamide (AIC) and its riboside. The excretion of hypoxanthine and xanthine was about six times higher in the patient. The excretion of AIC and its riboside was more than 20 times higher in the patient. Elevated excretion of AIC in this syndrome has also been reported by Newcombe (28). The excretion of adenine and adenosine were considerably lower in the patient than in the control (27). This could reflect the possibility that relative deficiency of adenine is a metabolic consequence of the metabolic abnormality in the patient.

## THE MOLECULAR DEFECT—HGPRT DEFICIENCY

The primary site for the expression of the mutant gene in this condition is the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). This enzyme converts the purine bases, hypoxanthine and guanine, to their respective nucleotides, inosinic and guanylic acids (IMP and GMP). A relatively nonspecific enzyme, it also utilizes xanthine as a substrate and the purine analogs 6-mercaptopurine, 6-thioguanine, and 8-azaguanine. These cancer chemotherapeutic agents require the presence of this enzyme and a conversion to nucleotides before they can be cytoactive. Deficiency in the activity of HGPRT in the Lesch-Nyhan syndrome was first reported by Seegmiller, Rosenbloom & Kelley (2) and has been confirmed by a number of investigators (29–32). The enzyme is normally present in all tissues of the body (33). In involved patients activity is deficient in every tissue in which it has been measured. It is most conveniently measured in the erythrocyte. Among normal tissues the levels of activity were higher in the basal ganglia than anywhere else tested. Throughout the brain, levels were four or more times those found in other parts of the body. Activity was absent in basal ganglia of a patient with the Lesch-Nyhan syndrome.

Quantitative assay of the enzyme in the erythrocyte has regularly revealed no activity. The more accurate the determination, the more it is clear that the values obtained in patients cannot be distinguished from zero. In fibroblasts or other tissues most assays show a little activity for HGPRT in patients. However, this may be an artifact of the method used. In most tissues a very active nucleotidase splits off the phosphate from IMP and GMP to form inosine and guanosine. To get reproducible results for HGPRT one must measure both inosine and IMP formed from hypoxanthine. However, the inosine formed could have come via the nucleoside phosphorylase reaction. This problem does not exist in the erythrocyte as there is no nucleotidase there, and HGPRT can be assessed more cleanly by measuring only IMP formed from hypoxanthine.

HGPRT is activated by magnesium ion and inhibited by the products of the reaction. GMP, guanosine diphosphate (GDP), and guanosine triphosphate (GTP) are more potent inhibitors than IMP of xanthine monophosphate (XMP) (34). The activity of the enzyme against hypoxanthine parallels that against guanine. All human variants studied have shown decreased activity against both substrates. The decrease is usually to the same degree.

HGPRT may be studied by electrophoresis on polyacrylamide disc gel. The pattern for HGPRT is a reproducible one in which there is a broad area of activity with suggestions of resolution into four subbands (35, 36). The mobility of the enzyme is much slower than that of adenine phosphoribosyl transferase (APRT), which forms a single sharp peak. Erythrocyte hemolysates from patients with the Lesch-Nyhan syndrome regularly yield an area of enzyme activity in this system (36). The activity is very low, and its mobility is faster than that of the normal enzyme. These observations indicated that the mutation which produced this syndrome is in a structural gene and specifies a protein with an altered primary structure. This conclusion has been confirmed by the demonstration in all of the patients studied that there is a protein which exhibits cross reactivity (CRM+) with antibody prepared against purified normal HGPRT (37–39).

Molecular heterogeneity within the Lesch-Nyhan syndrome has been demonstrated by McDonald & Kelley (40). They have reported a patient with an HGPRT with kinetic properties different from both the normal and the usual mutant enzyme. Erythrocytes from this patient have essentially no activity of HGPRT when the enzyme is assayed under the usual conditions. However, when assayed at very high concentrations of substrate the activity was normal. The $K_m$ for 5-phosphoribose-1-pyrophosphate (PRPP) and for hypoxanthine and guanine were different from normal, and the kinetic curve was sigmoidal when PRPP was used as the variable substrate. The authors concluded that at concentrations of substrate which obtain in vivo the enzyme in the patient would be expected to have little or no function. Further evidence for molecular heterogeneity was reported by Kelley & Meade (41) on the basis of studies of the enzyme in fibroblasts. They studied heat inactivation and product inhibition, obtaining three different patterns of response. Pattern 1 was of normal product inhibition and thermal stability. In pattern 2 product inhibition was normal and heat stability abnormal, and in pattern 3 both were abnormal. In two patients studied by Richardson and colleagues (42), one had normal $K_m$, but the enzyme was abnormally unstable to heat. In the other, the $K_m$ (hypoxanthine) could not be calculated because of the sigmoidal character of the curve; there was abnormal heat instability and activity was enhanced rather than inhibited by the product IMP. These observations provide further evidence for mutations in a structural gene. They suggest point mutations involving the substitution of a single amino acid rather than large deletions, nonsense mutations, or frame shifts. Clearly, there is considerable genetic heterogeneity at the HGPRT locus and a certain amount occurs in patients who have the classic Lesch-Nyhan syndrome. Further heterogeneity will likely be recognized.

The activity of APRT is regularly increased in patients with the Lesch-Nyhan syndrome (2, 32, 43). This may relate to diminished aging of the enzyme in the erythrocyte or to protection against decrease in activity with age. It could also result from a stabilization of the enzyme by PRPP; a stabilizing effect of PRPP has been demonstrated by Greene and colleagues on purified APRT in vitro (44). PRPP concentrations have been reported to be increased in the erythrocytes of patients with this syndrome (44, 45). APRT appears to be more stable in the circulation of patients than in normal subjects (44, 46, 47). However, this has been reported to be untrue in fibroblasts, even though these cells appear also to have increased concentrations of PRPP (48). The elevated concentration of PRPP in the fibroblast has been attributed to a decreased utilization in the absence of HGPRT (49).

Overproduction of purine and an increased rate of de novo synthesis has been related to an increase in the intracellular concentration of PRPP. It is thought that the glutamine-PRPP-amidotransferase is the rate-limiting step. It is not known whether the usual concentrations of PRPP are limiting for the reaction, but it has been assumed that increased concentrations would drive the reaction and hence the pathway. Erythrocyte concentrations of PRPP in the vicinity of $1 \times 10^{-6} M$ are certainly less than any established $K_m$ for the amidotransferase, although data on the human enzyme are unavailable. Establishing a drain on pools of PRPP by the administration of orotic acid (48, 50) decreases the rate of de novo synthesis. The decrease seen with the administration of adenine could also operate in this way, although direct action of AMP as a feedback inhibitor of the amidotransferase is thought to be the mechanism of this effect.

## GENETICS

The disorder is a disease exclusively of the male. Examination of several large kindreds with a number of involved boys indicated that transmission was always through the female to the male (51, 52). The pattern was that of an X-linked recessive trait. Application of the so-called grandmother test was consistent with this hypothesis.

Establishment of the deficiency of HGPRT activity in involved boys permitted the exploration of the genetics of the condition on a molecular level. In an X-linked recessive the fathers should have normal activity of the enzyme: this has uniformly been the case. The Lyon hypothesis specifies that the mothers should be heterozygotes who were mosaics containing two populations of cells, one completely normal and the other completely deficient. This has now been demonstrated by experiments in which fibroblasts growing in cell cultures derived from the skin were cloned (53, 54). Deficiency of HGPRT in the negative clones can be demonstrated radioautographically (53), by direct in vitro assay (54), or by electrophoretic assay (39).

The disease has never been seen in the female. It is certainly possible that it will turn up in an individual with an XO chromosomal constitution. Otherwise, our data indicate that the disease will be exclusively in the male. If the inactivation of the X chromosome were a completely random process, as proposed in the Lyon hypothesis, one would expect that there would be involved females. However, assessment of HGPRT activity in the erythrocytes and leukocytes of obligate heterozygotes for this condition has revealed a very different situation than in glucose 6-phosphate dehydrogenase (G6PD) deficiency, where the distribution of enzyme activities in a group of heterozygotes approximates 50%. In the mothers of patients with the Lesch-Nyhan syndrome, enzyme activities in the erythrocytes are completely normal in nearly every instance; furthermore, the few exceptions hardly deviate from normal. Leukocyte assays are also normal. These observations suggested that the process might not be random.

Information on this issue has been obtained through the study of a key kindred in which two types of G6PD were segregating as well as two types of HGPRT (55). In this family a number of boys with the Lesch-Nyhan syndrome were cousins, the sons of two sisters. It was assumed that the mothers were heterozygous for HGPRT, and this was proven by the assay of clones derived from fibroblast cultures of skin. Nevertheless, their erythrocyte and leukocyte activity of HGPRT was perfectly normal. Genetic analysis of the G6PD types of their parents and their children indicated that these two women were also heterozygous for G6PD; both had a G6PD AB genotype. However, repeated assay of their erythrocytes and leukocytes revealed only G6PD B. These observations indicated a clonal origin of the hematopoietic system. The data could be explained by nonrandom inactivation of the X chromosome containing the information for G6PD A and HGPRT$^-$. A more likely explanation would be random inactivation very early in fetal life followed by selection against the HGPRT$^-$ cell.

Cloning of cells derived from the skin of these women has indicated that the cell containing G6PD B is HGPRT-positive (HGPRT$^+$), while that containing G6PD A is HGPRT-negative (HGPRT$^-$) (56). Gartler & colleagues have studied this same family using hair follicles plucked from the scalp (57). Individual hair follicles are to a certain extent clonal. Assay of single follicles revealed some to be G6PD A or G6PD B. There were also some of the mixed AB type. In the meaningful A or B follicles, HGPRT assay always indicated the G6PD A cell to be HGPRT$^-$ and the G6PD B cell to be HGPRT$^+$.

Hemizygous expression of G6PD in a heterozygote has been reported by Fialkow and colleagues (58) in a patient with chronic myelogenous leukemia. It is of course less surprising to find a neoplasm such as a leukemia having a clonal origin. In carriers of the Lesch-Nyhan syndrome the entire hematopoietic system expresses the activity of only one of the X chromosomes.

Study of linkage of the gene for HGPRT with other genes along the X chromosome has been completed only in the case of the Xg blood group antigen. It has been found that there is no linkage between the two genes (59). Analysis of the Xg status of a group of heterozygotes for HGPRT negativity as well as of a group of women with chronic myelogenous leukemia led Fialkow and colleagues (60, 61) to conclude that the gene for the Xg antigen is not subject to lyonization, and that it does not inactivate normally with the X chromosome. This could mean that the gene is on or influenced by an autosome, or that parts of the X chromosome may escape inactivation.

The problem of hemizygous expression has complicated the process of heterozygote detection. This type of molecular diagnosis is essential for precise genetic counseling and for any approaches to control of the disease. Clearly, heterozygosity cannot be detected using the blood.

The presence of both cell types in cultures derived from skin permits the use of fibroblasts in culture for diagnosis. Heterozygosity has been recognized using radioautography of the whole mixed culture from the heterozygote (62). Direct-analysis skin culture can detect the presence of the gene in female siblings of patients and other relatives. However, autoradiography of the mixed culture is not reliable. A positive reading certainly indicates heterozygosity, but a negative one does not rule it out. The technique may thus be used to establish a relatively simple diagnosis if positive but indicate further testing if negative. The next step could be cloning, which is certainly reliable (53, 54, 56), but far from easy. The time and attention involved precludes its use as a routine method for the diagnosis of the carrier of the gene.

Pharmacological methods of cell selection (63, 64) provide an interesting and reliable answer to this problem. These methods soon became the approach of choice for heterozygote detection. It is still the cell-culture method of choice. The principle of selection takes advantage of the unique properties of these cells. HGPRT is required for the activation of 6-mercaptopurine (6MP) and related analog inhibitors of purine metabolism. These include 6-thioguanine (6TG) and 8-azaguanine (AG). Normal cells, containing HGPRT, are killed by these compounds while HGPRT$^-$ cells are unaffected. Thus, in the mixed populations of cultured cells from a heterozygote, this type of selective medium will permit the growth only of the HGPRT$^-$ cells. 6TG and AG are reliable agents for this purpose, and established concentrations reproducibly kill all HGPRT$^+$ cells. Selection for the normal cell in a heterozygote can also be done, but is not necessary for heterozygote detection. For that purpose, growth in AG or 6TG is enough. We believe that it is important to monitor the diagnosis with the chemical demonstration that the cells that grow in AG or 6TG are in fact HGPRT$^-$.

Selection for the normal cell depends on the fact that cells containing HGPRT will grow on hypoxanthine (H) when de novo synthesis is completely blocked in aminopterin-thymidine (AT) medium. Thymidine is used in the medium to establish a specific requirement for purines, for aminopterin inhibition of dihydrofolate reductase inhibits the addition of the methyl group to form thymidylate. HGPRT$^-$ cells cannot utilize hypoxanthine; when they are placed in HAT and de novo synthesis is blocked, they do not grow.

The assay of the enzyme in single hair follicles provides the fastest, easiest, and least expensive method of heterozygote detection (65–67). For routine use it is probably the best method and promises to supplant methods requiring cell culture. There are some technical problems. At least in our hands it is not always possible to obtain follicles. Women with long, fine, easily breakable hair are the most difficult. Cell culture methods may therefore be required if hair follicles cannot be obtained.

The hair follicle methods depend for validity on the viability of cell and enayme in each follicle. Gartler and colleagues (65) have tested this by splitting the extract obtained from each follicle and running both APRT and HGPRT. We do this electrophoretically and therefore get the simultaneous activity of both HGPRT and APRT on each single follicle. We believe that it is well to assay 20 to 30 hairs for diagnosis.

## AMNIOCENTESIS-INTRAUTERINE DIAGNOSIS

Expression of the defect in the fibroblast permits intrauterine diagnosis using fibroblasts cultured from fluid obtained by amniocentesis. Prenatal diagnosis was first made in twin boys (68). This pregnancy went to term, and the diagnosis was confirmed by assay of the enzyme in the infants and by their subsequent clinical course. A heterozygous female was also detected prenatally (69). More recently, intrauterine diagnosis of HGPRT deficiency has been made in two infants at risk for the Lesch-Nyhan syndrome, and pregnancy was interrupted (70, 71). The diagnosis was in each case confirmed by assay of the enzyme in tissues of the aborted fetus. These techniques therefore permit a decision on the interruption of pregnancy when the fetus has been diagnosed as involved, providing a certain amount of control of this disorder. Monitoring of pregnancy in this way is of course of enormous benefit to a family known to be at risk. It permits the carrier of the gene to have normal children. If all pregnancies are monitored, those permitted to go to term will be those with an established normal HGPRT. Most candidates for intrauterine diagnoses are mothers who have already delivered a boy with the syndrome. This limitation and the fact that this approach would probably increase some the number of heterozygotes indicate that this approach will do little to reduce the overall incidence of the disease in the world's population (72). This in no way minimizes the importance of the technique for families known to be at risk. It is excellent clinical practice, but more data are required for it to have public health importance. Diagnosis of heterozygosity in large numbers of female relatives in a family at risk helps; so do concerted efforts to diagnose all of the patients in a target population area. This might be done by screening large populations using the urate to creatinine ratio in the urine (73) or by one of the screening methods for HGPRT that permit the use of a drop of blood dried on filter paper (30, 31). However, for a decisive impact on the disease in a large population, detection of all of the heterozygotes would be required, an impossibility with current methods.

## SOMATIC CELL GENETICS AND BIOLOGY

An interesting phenomenon has been observed in cultures containing HGPRT-deficient cells and called metabolic cooperation (74). If HGPRT$^-$ and HGPRT$^+$ cells are grown together and then radioautographed using $^3$H-hypoxanthine, all well-separated cells are clearly positive or negative. However, in a mass of confluent cells the detection of negative cells is difficult. Determination is clearer on inspection of sparse populations of cells in which there is only occasional contiguity. It can then be seen that wherever a negative cell touches a positive one it develops a positive reaction, first in the area nearest the point of contact. Thus, something is transferred across the borders of contiguous cells that permits the formation of the nucleotide and its incorporation into RNA. This phenomenon obviously creates problems in heterozygote detection in unselected cell cultures.

The ability of cells lacking HGPRT to grow in selective media containing AG or 6TG and their inability to grow in media containing HAT provide important tools with many uses in the in vitro study of human cells. One possibility is the study of mutation. This system has been stated to be the "best candidate yet for experimental studies of mutation in human genetic material" (75). It can be used to study spontaneous rates of mutation and in the study of compounds which should be tested for mutagenesis, because they are either to be added to food or to some other aspect of our environment.

New mutants occurring spontaneously in vitro have been selected using AG from normal human diploid male cells (76). These HGPRT$^-$ cells behave exactly like those derived from patients with the Lesch-Nyhan syndrome.

The system is also ideal for the study of hybridization (77). For instance, if one starts with cells derived from patients with the syndrome, the cells will not grow in HAT. Hybridization with a normal cell derived from man or mouse will permit growth in HAT. The parental cell containing the gene for HGPRT$^-$ can then be selected against by growth in AG, leaving only the hybrid cells. Similar studies on hybridization have used cells lacking thymidine kinase, which also will not grow in HAT. Selective loss of human chromosomes in human-mouse or human-hamster hybrids permits localization of such an enzyme to a single chromosome.

An important extension of this approach has been reported by Harris and colleagues (78), who corrected HGPRT deficiency in cultured mouse fibroblasts by fusion with nuclei of chicken erythrocytes in the presence of inactivated Sendai virus. When the chicken erythrocyte nucleus is introduced, it resumes DNA and RNA synthesis and determines the synthesis of chicken proteins. With mitosis of the heterokaryons, the erythrocyte nuclei can no longer be seen but are fragmented in what has been called chromosome pulverization, a method that seemed ideal for incorporating small pieces of genetic material into somatic cell nuclei. Culture of the heterokaryons in HAT selected for those containing the HGPRT. The clones formed contained HGPRT, and the electrophoretic mobility of the enzyme indicated it to be chicken in origin. The amounts of genetic material incorporated were so small that no chicken chromosomes could be seen and there were no chicken cell surface antigens.

## THE PATHOGENESIS OF CEREBRAL MANIFESTATIONS

It is of enormous importance that we develop an understanding of the pathogenesis of the neurological and behavioral aspects of the syndrome. This could provide chemical insights into the function of the nervous system and into behavior. How the biochemical abnormalities interact to produce clinical manifestations is not known.

Ultimately, the fundamental cause is the lack of activity of HGPRT. However, individuals with a variant HGPRT that function at a level of 5% of normal activity have no neurological and behavioral abnormalities, although they do overproduce purine and have all of the consequences of hyperuricemia itself. The first consequence of a deficiency of HGPRT might be a shortage of nucleotides, which might interfere with feedback inhibition of PRPP-amidotransferase and provide a mechanism for the overproduction of purine de novo. It has been our operating hypothesis that the overproduction results in the production of some purine products which produce the cerebral and behavioral manifestations of the disease.

This hypothesis is consistent with experience with phenylketonuria, where dietary therapy provides the best in vivo evidence for cerebrotoxic byproducts of an enzymatic defect. The fact that involved infants with the Lesch-Nyhan syndrome are normal at birth and develop normally for the first ten months of life is also consistent with a toxic theory of the pathogenesis of cerebral manifestations. There is a little more evidence. It has been possible to produce an animal model for self-mutilation by the administration of methylpurines to rats and rabbits (79, 80). In the rat the dosage required is of the order of an $LD_{50}$, but in the rabbit smaller doses are effective. Caffeine (1,3,7-methylpurine) is effective, and theophylline (1,3-methylpurine) is even more effective. Theobromine (3,7-methylpurine) is inactive. Therefore, it appears that the 1-methyl or 1,3-methyl structure is important. Furthermore, the effect is not simply that of central nervous system stimulation. The fact that theophylline is an inhibitor of diesterase raises the possibility that cyclic AMP may be involved in the mechanism of self-mutilation.

The problem may also be studied using cultured cells in vitro. A requirement for adenine for the growth of cells from patients with the Lesch-Nyhan syndrome has been established by Felix & DeMars (81). Normal cells can apparently make all the purines they need. These studies also established a requirement for about 50 times as much folic acid for optimal growth as in normal cells. These observations came about using a structured medium, while apparently the media used by most of us contained enough purine, folic acid, or both, that these requirements were not apparent. They are consistent with observations of van der Zee and colleagues who reported that the megaloblastic anemia sometimes seen in patients with this syndrome is responsive to adenine (20). It is also consistent with the presence of lower than normal amounts of adenine in the urine of a patient (27).

## OTHER DISORDERS OF PURINE METABOLISM-MOLECULAR HETEROGENEITY IN HGPRT

A number of patients have been described who do not have the Lesch-Nyhan syndrome but who have abnormal AGPRT activity. Quantitative assay always yields an activity greater than zero. These mutations are also on the X chromosome (82). In a kindred studied by Henderson and colleagues (83), three affected males produced seven sons, none of whom was affected. This strongly supports X-linked inheritance. Involved patients also have increased activity of erythrocyte APRT, and to the same degree as patients with complete deficiency of erythrocyte HGPRT.

The activity of HGPRT in the erythrocytes of involved patients varies with the kindred. In any one kindred the degree of activity is virtually identical in all involved members. Most patients described have had from about 5 to 15% of normal activity (82, 84); a few have had less. In some the stability of the enzyme to heat has been similar to that of the normal enzyme; in some there has been altered heat stability (82, 84). In every instance reduced activity against hypoxanthine has accompanied reduced activity towards guanine. The percentage reduction in activity against one substrate has often been identical to that to the other. However, this has not always been the case, which is consistent with what one might expect for a spectrum of changes in the primary structure of the enzyme protein. Electrophoretic analysis in one kindred revealed an HGPRT with a mobility slightly faster than that of normal HGPRT and about the same as the mobility of the enzyme from patients with the Lesch-Nyhan syndrome (85).

A man has recently been studied by Benke and colleagues (86) in whom hyperuricemia, hyperuricosuria, and overproduction of purine de novo were associated with normal activity of HGPRT. However, cells derived from this patient grew in AG medium, a property previously observed only in HGPRT deficiency. A detailed kinetic study has indicated that the HGPRT is very abnormal in this patient, even though the abnormality is not detectable under the conditions of substrate saturation usually used for routine assay of the enzyme (87).

A number of mutations at the HGPRT locus have been defined. It seems likely that this is only a beginning and that a considerable molecular heterogeneity will ultimately be documented.

Patients with partial deficiency of HGPRT are generally found among male patients with clinical gout. These patients have large amounts of uric acid in both blood and urine. They may present with renal stone disease in childhood (82) or later. They may develop urate nephropathy; this too may occur in childhood, but not usually. Acute attacks of gouty arthritis and tophaceous deposits of urate generally appear first in the adult even though the metabolic abnormality has been present from birth. In this as in other forms of hyperuricemia many years of elevated concentrations of urate seem to be required before the onset of the arthritic manifestations that are ultimately the hallmark of gout.

A number of these patients have had some abnormality of the nervous system (84). This has been occasionally taken the form of mild mental retardation, or of spinocerebellar degeneration. It is not at all clear that these associations are anything but chance. Most patients with spinocerebellar degeneration have no abnormality of HGPRT or of uric acid, and most patients with partial deficiency of HGPRT have nothing wrong with the central nervous system. Behavioral abnormalities and self-mutilation have been conspicuous by their absence. One of our young adult patients was a rather vigorous fingernail biter (82), and like many nailbiters also bit some of the areas of the skin about the nails. Benke's patient (86, 87) has displayed some antisocial behavior, and has been in jail.

Heterozygotes for these variants have not been studied extensively enough to permit generalization. Most of those we have studied have had normal activity of HGPRT in the erythrocyte, as in those carrying the gene for the Lesch-Nyhan syndrome. However, this is not always true. Some heterozygotes for partial activity have partial activity of HGPRT in their erythrocytes, more than their hemizygous brothers but considerable less than normal (82, 85). These observations suggest that selection against cells carrying variant HGPRT depends on the degree of HGPRT deficiency. Data reported by Emmerson & Wyngaarden (88) have been recalculated by DeMars and presented in support of such a hypothesis. The mother of two boys with the Lesch-Nyhan syndrome had, as usual, normal erythrocyte activity of HGPRT. Her cumulative incorporation of glycine into urinary urate was somewhat greater than normal, but the size of the urate pool and the rates of urate production were normal. In contrast, in two mothers of patients with partial defects, erythrocyte activities of HGPRT were 44 and 17% of normal. Urate production was considerably above normal, and the urate pools were larger. Incorporation of labeled glycine into urinary urate was about four times normal. These findings imply that the complement of normal cells in these women could not supply what is required to maintain a normal rate of purine synthesis in the liver and other metabolically active organs. The data suggest that the selection observed in the hematopoietic system in heterozygotes for the Lesch-Nyhan syndrome probably involves most tissues of the body. In the partial defect, variant cells may be present throughout the body. No data are available on the central nervous system.

Review of the literature from 1823 to the description of the Lesch-Nyhan syndrome revealed 15 patients less than 10 years of age with hyperuricemic disorders (1). Some of these may, of course, have had HGPRT deficiency and some may have had glycogen storage disease, but there was no recorded evidence of this latter condition. Some may have had normal HGPRT activity because five of them were girls.

Hyperuricemic children are now being turned up in increasing numbers. Some of them have normal HGPRT activity. We reported a boy with hyperuricemia, increased urinary urate, and an increased rate of incorporation of $^{14}$C-glycine into urate whose HGPRT in both erythrocytes and fibroblasts was normal (89). This boy had a number of other abnormalities which may or may not be related. He was mildly mentally retarded, had small dysplastic teeth, failed to cry with tears, and displayed autistic behavior.

A girl has been reported from the Netherlands in whom there was increased production of uric acid, choreoathetosis, and self-mutilation (90). HGPRT has not been assayed. However, the kinetics of the incorporation of $^{14}$C-glycine into uric acid were so different from those of patients with HGPRT deficiency that we would expect the activity of that enzyme to be normal.

We and others are studying a number of patients in whom hyperuricemia and increased urinary urate accompany normal activity of HGPRT. In our view the variety of hyperuricemic disorders has only begun to be defined.

## TREATMENT

There is currently no treatment capable of affecting the central nervous system manifestations of the Lesch-Nyhan syndrome.

Those aspects of the disease directly related to uric acid itself, the manifestations which patients with this syndrome have in common with adults with gout, are effectively managed using allopurinol. The drug is therefore highly effective in the other patients we have diagnosed who have overproduction hyperuricemia with either normal activity of HGPRT or with partial deficiency of HGPRT. The oral administration of allopurinol in a dose of 200 to 400 mg a day causes a reduction of plasma and urinary levels of uric acid and a concomitant increase in the oxypurines, hypoxanthine and xanthine (24, 25). In controls and in adults with gout and normal activity of HGPRT, the total excretion of oxypurines, i.e. the sum of uric acid, xanthine, and hypoxanthine, is less after treatment with allopurinol than under control conditions. In patients with this syndrome this decrease in oxypurine excretion is not seen (24, 91). It is our view that this represents the importance of the reutilization of hypoxanthine via HGPRT rather than an effect of allopurinol ribotide on the rate of synthesis of purine de novo. There are data in control animals that indicate that the decrease in total oxypurine excretion after allopurinol can be accounted for by reutilization of hypoxanthine and xanthine (92). In the absence of HGPRT this reutilization would not be possible; the total excretion of oxypurines should not fall.

Following treatment with allopurinol and effective reduction of uric acid levels, we have seen rapid resorption of tophi, as well as improvement in renal function, as measured by the concentrations of urea and creatinine in the blood. Thirst, a prominent symptom in many patients, may decrease with allopurinol therapy. Crystalluria, hematuria, stone formation, and concomitant urinary tract infection may certainly be prevented by treatment. We have not seen stones resorb, but rather they have decreased in size and have been passed. Large radioopaque stones may contain too much calcium to be affected by therapy. Arthritis and nephropathy should be preventable by early and continued treatment.

There have been some encouraging evaluations which suggested that allopurinol might be effective in the cerebral and behavioral aspects of the disease (93). However, we have had experience with its use in a number of patients with established symptoms and have seen no changes that could not be attributable to improvement in general condition. Furthermore, we have followed two patients (94, 95) who were diagnosed in the first days of life and effectively managed with allopurinol from the point of view of uric acid concentrations; both developed the syndrome undistinguishable from others that we have studied.

Xanthine stones have been observed in patients with this disease (96, 97) during therapy with allopurinol. However, this complication is rare, even with massive doses of the drug (24). If the patient with HGPRT deficiency behaved metabolically like other individuals, xanthine stones would be a common complication of allopurinol therapy. Control individuals treated with large doses of allopurinol and virtually complete inhibition of xanthine oxidase excrete essentially all of their purine as xanthine, as do xanthinuric individuals who have a genetically determined defect in xanthine oxidase. However, patients with HGPRT deficiency are unable to reutilize hypoxanthine and most of their purine leak appears in the urine as hypoxanthine (24). As the block is increased with increasing dosage of allopurinol, these patients become increasingly hypoxanthinuric. While xanthinuria is even more likely to lead to stones than uric aciduria, hypoxanthinuria is much less likely to do so because hypoxanthine is much more soluble than these other two purines. These factors, along with the fact that generally the purine load which was all in uric acid prior to treatment is now directed to two purines, greatly decrease the likelihood of stone formation with treatment.

Probenecid and other uricosuric agents have been used in the management of this disease (1, 7). In most cases in which renal disease has not been established this type of therapy does lead to a reduction in the concentrations of uric acid in the blood. However, it has no favorable effect on the cerebral aspects of the disease. From the point of view of the kidneys, these patients are already processing an enormous amount of renal urate. A uricosuric agent could lead to renal shutdown and death. We feel that uricosuric agents are contraindicated in patients with HGPRT deficiency. Alkali therapy with sodium citrate or sodium bicarbonate is effective in many patients who drink sufficient water to prevent crystalluria and stones and to maintain a normal level of urea nitrogen in the blood. However, this form of management is much more difficult than allopurinol therapy, particularly at times of intercurrent illness.

A number of approaches have been made to the therapy of the cerebral manifestations of this syndrome, and a few have now been reported. Ghadimi (98) reasoned from observations on amino acid analysis that there might be substrate depletion of glutamine in the course of the overproduction of purine de novo. He therefore fed monosodium glutamate in large doses and reported improvement not only in appetite and weight gain but also in scissoring and in the urge for self-mutilation. We have doubted that this treatment had any effect on the cerebral problems of his patient. It is always difficult to extrapolate from uncontrolled observations. Ghadimi is quite right in pointing out the extreme degree of emaciation one sees in these patients. We have probably not emphasized it sufficiently, but these patients can die of inanition. However, it is a consequence, not a cause, of their athetosis, difficult feeding, and vomiting. All of the babies we have followed have been quite well nourished before they began to degenerate. Inanition has followed much later. There is no question, however, that these patients can improve markedly with improvement in nutrition as would anyone who is starving. We have seen a 12-year-old patient admitted in a moribund condition, bedridden for years, and with what appeared to be contractures, who on therapy doubled his weight, gained complete mobility, and also decreased his spasticity and self-mutilating activity. The therapy in this case was simply excellent nursing care, painstaking feeding, and attention. For these reasons we feel that therapeutic studies should be looked at closely, controlled if possible, and preferably be done early in the patient's life. We have nevertheless tried glutamic acid treatment as reported by Ghadimi in a patient with this syndrome, documenting behavior on film before and after. We could see no effect.

Benke & Anderson (99) have reported initial observations on twins diagnosed *in utero* and treated promptly after birth. The therapeutic plan was based on the finding that HGPRT-deficient cells need adenine or excessive amounts of folic acid for growth (81). One twin was treated with adenine and both received folic acid and bicarbonate. Both boys developed typical cerebral manifestations of the disease. The therapy is rational. Inhibition of rates of de novo synthesis and areas of purine interrelations remain a promising approach for therapeutic exploration.

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# GENETICS OF CONGENITAL HEART DISEASE 7059

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The study of this subject originally proved difficult for a variety of reasons including relative imprecision of clinical diagnosis, lack of adequate animal homologues, and absence of any well defined biochemical or enzymatic correlations. In the first edition of his textbook, Wood (1) stated with that clarity and "incisiveness and perhaps dogmatism" (2) which makes his writing so vivid a memorial of his life: "Congenital anomalies account for one or two percent of all cases of organic heart disease. They are not hereditary and they are rarely familial."

Familial incidence studies involving 500 or more probands were undertaken by various groups (3–6). All showed a recurrence rate in sibs of between 1.5 and 5%, compared with the now accepted incidence in the population as a whole of between 0.6 and 0.8% (7). Nora et al (8), using the family study of atrial septal defect as a model to evaluate the hypothesis of multifactorial inheritance in congenital heart disease, found a recurrence rate of 3.2% in sibs and 2.6% in offspring of probands. He emphasized the closeness of the observed and expected findings assuming the multifactorial hypothesis. He reexplored the previously somewhat confusing twin data (9) and emphasized the significance of genetic environmental interaction in the etiology of congenital heart disease (10).

Previous summaries of the different approaches used in the elucidation of genetic aspects are available (11–17). Papers presented at the Fourth Conference on the Clinical Delineation of Birth Defects are currently being published as a monograph (18) and give the interested reader extensive references to all the recent papers on familial aggregations of specific defects and the association of cardiac anomalies and clinical syndromes. In the present discussion we will attempt to outline the current status of ongoing studies of specific cardiac defects. Cardiac anomalies associated with heritable disorders of connective tissue or neuromuscular diseases are omitted from detailed consideration.

## TYPES OF CONGENITAL CARDIOVASCULAR ANOMALY

The lack of any generally accepted classification of cardiac malformations has made logical consideration of the genetic aspects more difficult. The use of a list of lesions "most frequently seen" in a clinic is undesirable because of the effects of differential mortality. The classification we are using here is tentative and based on VanPraagh's concept of the segmental approach to cardiac development (19).

## ANOMALIES OF VISCEROATRIAL SITUS AND BULBOVENTRICULAR LOOP

Since Cockayne's demonstration of an increased incidence of parental consanguinity in true mirror image dextrocardia (20), it has been generally agreed that this malformation is transmitted by an autosomal recessive mode of inheritance. Campbell (21) suggests that isolated levocardia is similarly inherited, but more data seem to be needed on this and on the complex types of cyanotic dextrocardia encountered in populations attending congenital heart clinics.

Anomalies of the bulboventricular loop may result in ventricular inversion or corrected transposition. To our knowledge there have been no family studies recorded on this particular group. A most interesting study by Stevenson & Huntley (22) on the offspring of phenylketonuric mothers disclosed a variety of cardiac lesions including extreme hypoplasia of the left ventricle. Although the way in which the persisting biochemical aberration of a phenylketonuric mother may affect the fetal developing heart is unknown, this study affords perhaps the most dramatic human example to date of genetic environmental interaction.

## CONOTRUNCAL ANOMALIES

A number of familial aggregations have been reported in tetralogy of Fallot (TOF) and in other conotruncal anomalies (23–26). Recurrence rates in sibs (10) and offspring (27) have been ~2.5 to 3%, and approximately two thirds of the recurrences are concordant.

The most interesting recent work on conotruncal anomalies is that of Patterson (28). Working with the keeshond, which he had previously determined to have a breed-specific prevalence rate of congenital heart disease of 76 per thousand, he was able by cross breeding to produce 58 dogs with cardiovascular malformations. Virtually all of these had either tetralogy of Fallot or some other anomaly of the heart compatible with hypoplasia or maldevelopment of the conus septum. Of particular interest was his demonstration that in some cases the defect was a "forme fruste;" for example, an aneurysmal bulging of the conus septum detectable only on careful angiography or autopsy examination. The results obtained were not clearly attributable to either a single gene with reduced penetrance or to a true multifactorial system. The implications of this work and particularly the opportunity to study cardiac maldevelopment in its early stages are most exciting.

Despite its relative frequency as a cardiac anomaly, TOF is rarely associated with any of the recognized syndromes or trisomies, being, for example, rare in Down's syndrome (29). It has, however, been recorded in association with the syndrome of thrombocytopenia with absent radius (30) and in the DiGeorge syndrome (31), among others.

Complete transposition of the great arteries is even more rarely associated with other defects and Mitchell (17) has suggested that some hormonal imbalance early in pregnancy may lead to the conal inversion which is the embryonic basis of the anomaly.

## DEFECTS OF CARDIAC SEPTATION

These include defects of the atrial and ventricular septa and the endocardial cushions.

*Atrial septal defect* (*ASD*).—This anomaly, as might be expected from the relative ease of diagnosis and the longevity and relative fertility of the affected patients, has been the most frequently studied for familial aggregations (32–35). In most families the incidence data are compatible with multifactorial inheritance and recurrence rates of 2–4% in first degree relatives are recorded.

However, ASD of the secundum type is the usual cardiac finding in the Holt-Oram syndrome (36, 37) in which absence or digitalization of a triphalangeal thumb is the most striking external manifestation of maldevelopment of the radius. This syndrome is inherited as a dominant with varying degrees of expressivity. It has been suggested that ASD with prolonged atrioventricular conduction may also on occasion show a dominant pattern of inheritance even in the absence of skeletal anomalies (38).

*Ventricular septal defect* (*VSD*).—The ventricular septum undergoes closure over a long period of intrauterine life and the importance and frequency of spontaneous postnatal closure is only now beginning to be appreciated. Both clinical and experimental work suggest the importance of genetic and environmental influences on the developing septum (39–41). The incidence of VSD in the offspring of probands was found by Nora et al (42) to be 3.7%.

*Endocardial cushion defects* (*ECD*).—Both the ostium primum and AV canal type of defect are frequent in Down's syndrome (29) and these unfortunate infants seem also to have a greatly increased risk of the premature development of pulmonary vascular obstructive disease. A form of endocardial cushion defect, the single atrium, is rather characteristic of the Ellis-van Creveld syndrome, a well-known recessive disorder (43). Emmanuel et al (44) did one of the few family studies of atrioventricular defects and found identical cardiac defects in one out of eight first degree relatives, giving a calculated heritability of 45%, lower than that calculated for simple secundum ASD (70%).

## ATRIOVENTRICULAR VALVE AND SEMILUNAR VALVE ANOMALIES

Past family studies had shown significant variation in the recurrence risk rates for pulmonary stenosis of the valvar type (PSV). It now seems highly likely that centers recording a recurrence risk greater than 2% had inadvertently included some examples of Noonan's syndrome (45). The full spectrum of this disorder is still being disclosed. For our present purposes the major features are hypertelorism, neck webbing, and other similarities to the Turner phenotype, but with a normal chromosome component. Inheritance is by an autosomal dominant transmission with varying expressivity, and the pulmonary valve tends to be dysplastic rather than the usual dome-shaped valve seen in isolated stenosis in otherwise normal subjects. Although other defects have been recorded, the absence of coarctation in this syndrome and its high incidence in Turner's or XO syndrome, and the fact that aortic valvar stenosis may occur in both lesions is an interesting and still unexplained phenomenon.

A strong familial tendency clearly seems to be present in congenital prolapse of the posterior leaflet of the mitral valve (46). Now that this defect can be so well identified by noninvasive techniques, large scale family studies seem warranted. The present concept is that the defect is a fibromyxomatous degeneration of the leaflet similar to that seen in Marfan's syndrome, but without other stigmata of that disorder; an autosomal dominant mode of inheritance is currently being postulated.

Semilunar valve anomalies have also been recorded in association with multiple lentigines (47) and congenital deafness and arrhythmia.

### SUPRAVALVAR (PERIPHERAL ARTERIAL) ANOMALIES

Since the recording of supravalvar aortic stenosis in grandmother and grandson by our group (48), a vast literature has burgeoned on both supravalvar aortic and pulmonic stenosis. Most of the interest has been in the possible relation of these defects to the so-called "hypercalcemia syndrome" (49, 50). Many extensive family trees are available showing that at least in some instances supravalvar lesions appear to be inherited by an autosomal dominant mode of transmission (51). The relationship of these families to patients with mental retardation and abnormal facies is not clear, but at the moment recurrence risks should be considered high.

### CARDIOMYOPATHY AND CONDUCTION DEFECTS

Hypertrophic subaortic stenosis has long been recognized as familial in many instances and is probably inherited as a dominant with delayed expressivity (52). For a detailed review of the other congenital cardiac myopathies and familial conduction defects, the reader should consult Reference 18.

## SUMMARY

Before attempting to assess risk of recurrence in the first degree relatives of a proband, it is essential to have a precise anatomic diagnosis and a detailed family tree. In the absence of any other affected family member and the absence of any of the major syndromes described above, the overall risk of recurrence of the common cardiac malformations can be confidently predicted to be less than 5%. Although data are inadequate at present, the presence of another affected first degree relative probably increases the risk of recurrence to between 15 and 20%.

The clinician needs to be constantly aware of the concept of genetic heterogeneity and to recognize that in familial and genetic studies precise anatomic definition of both cardiac and extracardiac anomalies is essential.

Before counseling a patient with aortic stenosis, for example, it is clearly essential to define the exact site of the stenosis—supravalvar, valvar, or subaortic. Before counseling a patient with atrial septal defect, it is essential to look for minor anomalies of the hand, both in the proband and ideally in close relatives. Before counseling a patient with pulmonary valvar stenosis, search should be made for stigmata of Noonan's syndrome.

Although most of our knowledge of this subject remains on an empirical basis rather than at a cellular level, recent developments in animal work and more careful study of familial aggregations give hope for a more sophisticated understanding.

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# CHROMOSOME IDENTIFICATION[1]

7060

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## INTRODUCTION

Human cytogenetics has undergone two revolutions in the last 15 years. After the technical innovations introduced by Hsu in 1952 (1), the correct number of human chromosomes was shown to be 46 by Tjio & Levan in 1956 (2). Within three to four years, a new field of human biology was opened, and most of the common chromosome aberrations were described and categorized, the first of these being the identification of Down's syndrome or mongolism as trisomy 21 by Lejeune's group in 1956 (3).

During the succeeding ten years, many cases with numerical or structural aberrations were described, all using the methods of identification of chromosomes outlined in a series of three conferences (4–6). Cytogeneticists soon became aware of the limitations of chromosome identification owing to inability to distinguish chromosomes within morphologically similar groups, as well as the difficulty of recognizing the size and position of structural rearrangements. Some improvement in identification was brought about in the early 1960s by the introduction of autoradiographic analysis of time differences in DNA synthesis of different chromosomes (7). The last two years, however, have seen a second revolution in cytogenetics caused by the discovery of several methods which permit the accurate identification of each chromosome and its parts.

## NEW TECHNIQUES

*Quinacrine fluorescence (Q-banding).*—This first method developed from the observation of Caspersson et al in 1968 (8) that specific chromosome regions become fluorescent after staining with quinacrine mustard. By 1970, these investigators were able to show that each human chromosome developed specific patterns of fluorescent bands which could be analyzed either photographically (9) or by photoelectric measurements along each metaphase chromosome (10). A number of other laboratories rapidly confirmed these findings (e.g. 11). Although Caspersson initially suggested (8) that the regions of fluorescence may be rich in guanine and cytosine because of preferential binding of alkylating substances

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such as mustards to guanine, the observation by several authors (e.g. 11, 12) that quinacrine dihydrochloride produces the same fluorescent patterns as does quinacrine mustard refuted this speculation. In fact, recent studies (13, 14) indicate that quinacrine binds preferentially to chromosome regions high in content of adenine and thymine, and that the intensely fluorescing regions are essentially identical to those chromosomal segments which replicate their DNA late in the S-period (13, 15, 16). There are, however, a number of exceptions to this occurrence. The possible meaning of this correlation and its exceptions will be discussed later when we consider the possible significance of the various new techniques for the elucidation of chromosome structure and function.

A photograph and schematic karyotype of the fluorescent patterns are shown in Figure 1. In addition to the individual chromosomal patterns, two important features observed with this technique are the intense fluorescence of the distal two thirds of the Y chromosome (17), and the variation of intensity and size of certain fluorescent regions (designated by *p* in Figure 1) which vary polymorphically between individuals but are inherited from parent to child.

Q-banding has already proven useful in resolving a number of clinical and research problems. For example, variant and abnormal human Y chromosomes in males and phenotypic females have been accurately characterized (18, 19). Chromosomes 21 and 22 have been distinguished from each other, permitting identification of the chromosome trisomic in Down's syndrome as 21 (20), while the partly deleted Philadelphia ($Ph_1$) chromosome characteristic for chronic myeloid leukemia has been identified as 22 (21, 22). A number of numerical and structural aberrations in chromosomally abnormal individuals have been accurately characterized (16, 23). Abnormal chromosomes have been identified in lymphoid cell lines from patients with Burkitt's lymphoma and other diseases (24, 25). Accurate identification of human chromosomes in human-mouse hybrid cells has resulted in correct assignment of specific genes to specific chromosomes (26). The presence of the polymorphic variants shown in Figure 1 have even been utilized in a paternity investigation (27).

Perhaps the most important use of this technique results from the finding (28) that in most individuals who have a Y chromosome, the fluorescent portion of the Y can be visualized after staining nondividing cells such as those obtained from the buccal mucosa (Figure 2) or peripheral blood leukocytes. This has permitted screening of groups of individuals for the presence and number of Y chromosomes (two Y chromosomes result in two fluorescent bodies in the nucleus), as well as identifying spermatozoa which carry either one or two Y chromosomes (29–31). However, attempts at sexing amniotic fluid cells by this method, even when combined with studies of sex chromatin, are not sufficiently accurate (32), and prenatal sex determination preferably should be performed by karyotype analysis.

Finally, the fluorescent technique has proved to be useful in identifying individual chromosomes in other species, including primates (33), mice (34), and plants (35).

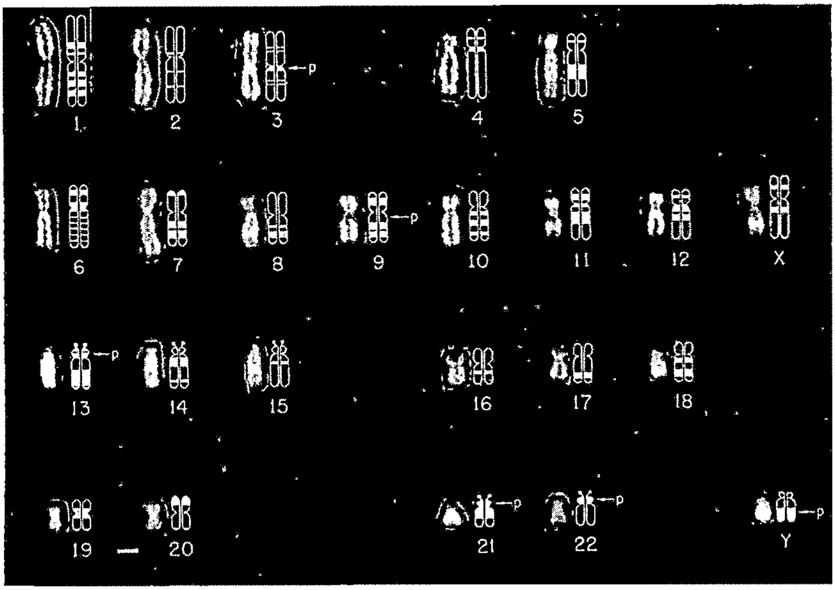

FIGURE 1. Chromosomes and schematic banding patterns after staining with quinacrine and photographed with fluorescence microscope. *p* indicates areas of individual inherited variation.

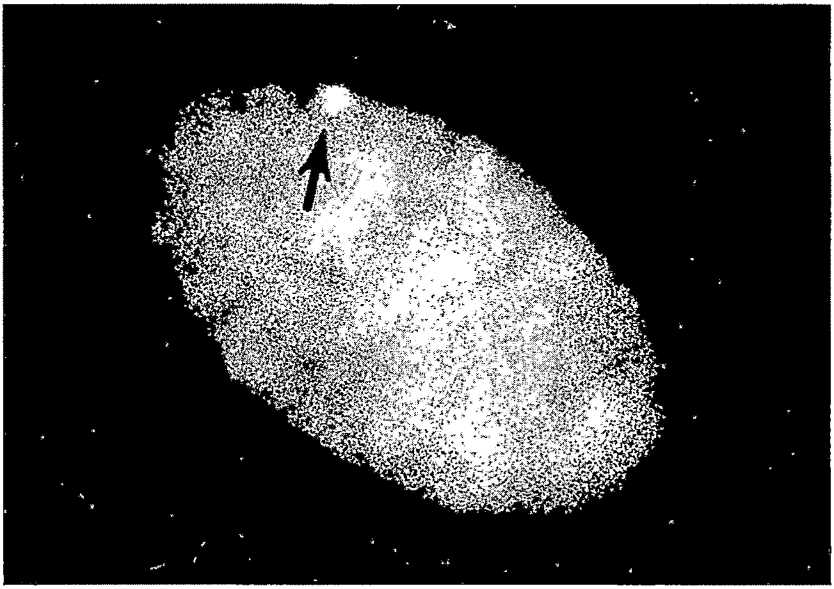

FIGURE 2. Buccal mucosal cell from normal male stained with quinacrine and photographed with fluorescence microscope. Arrow points to fluorescent Y body at periphery of nucleus.

*Staining of repetitive DNA* (*C-banding*).—In 1971, Arrighi & Hsu (36) described a method which resulted in intense staining with Giemsa of the centromeric regions of chromosomes, as well as of the distal two thirds of the long arm of the Y (Figure 3). This method was based on the observation of Pardue & Gall (37) that the fraction of DNA consisting of highly repetitive copies of short segments [originally characterized as satellite DNA by Britten & Kohne (38)] renatures more rapidly than other types of DNA after alkaline denaturation. Treatment of metaphase chromosomes by this technique of denaturation, followed by renaturation in neutral salt solutions, produces intense Giemsa staining of the renatured portions of the chromosome. As can be seen in Figure 3, these portions exist primarily in the centromeric regions, the long arm of the Y chromosome, and in the areas of secondary constriction characteristic of the long arms of chromosomes 1, 9, and 16 adjacent to the centromere. This technique has proven useful in identifying chromosome 21 in Down's syndrome (39), and in detecting polymorphisms of the areas of secondary constriction and of centromeric regions (40). Similar areas of repetitive DNA have been found in other species (41, 42). In electron microscopic studies, these regions are characterized by numerous interarm fibers (43).

*Giemsa banding* (*G-banding*).—During the last year, a number of techniques have been described, all of which result in characteristic banding patterns specific for each chromosome when stained with Giemsa after the appropriate treatment (Figure 4). These banding patterns can be achieved by treating the fixed metaphase chromosomes on the slide by consecutive exposure to sodium hydroxide and Sorenson buffer at 59°C (44); by exposure to certain buffered salt solutions at various temperatures (45, 46); by staining with Giemsa at pH 9 (47); by a modification of the repetitive DNA technique discussed above (48); or by treating with proteolytic enzymes including pronase (49), chymotrypsin (50), and trypsin (51, 52). Similar bands can also be produced by treatment with urea (53), or with cold treatment in the latter part of the culture period (54). The latter technique derives from an observation by Darlington & LaCour in 1938 (55) of segments of reduced stainability in the chromosomes of several organisms following treatment of dividing cells at a temperature of 0–3°C. Comparison of Giemsa banding with the patterns produced by quinacrine fluorescence has shown identity for all bands except those associated with secondary constrictions, other polymorphic areas, and to some extent, the Y chromosome (56, 57). The most reproducible and fastest of the G-banding techniques available is a modification (58) of the trypsin method described by Seabright (51). We use 0.1% trypsin (Difco 1:250) in phosphate-buffered saline at pH 7 for 45 sec at 20°C. The slides are rinsed by brief dipping in 50% ethanol: distilled $H_2O$ and then 100% ethanol. They are stained for 2 min in Giemsa (G. Gurr) diluted 1:5 in pH 6.8 phosphate buffer. If the slides are less than two weeks old, they are heated overnight in a 60°C oven.

Electron microscopic observation (59) of trypsin-treated chromosomes reveals a release of protein from chromatin fibers. The Giemsa banding techniques

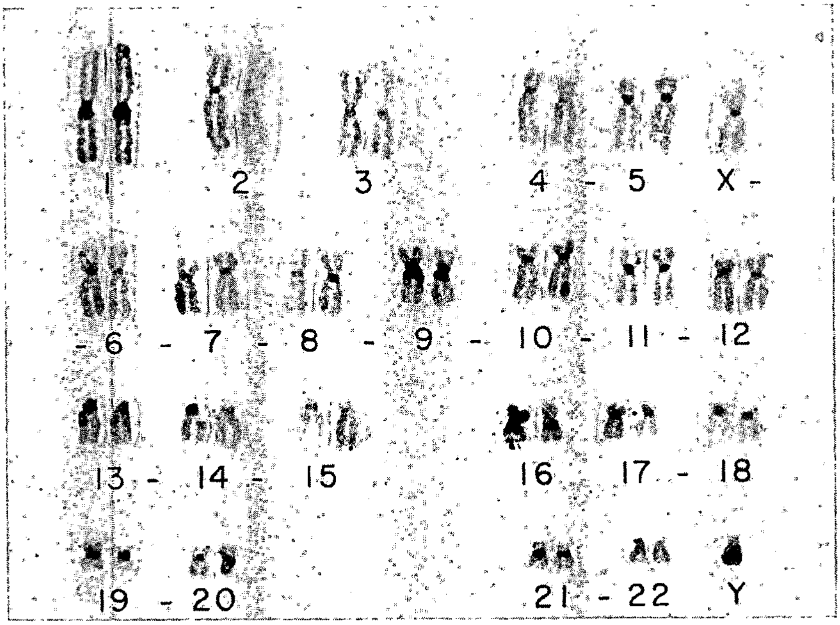

FIGURE 3. Karyotype from normal male stained for repetitive DNA by method of Arrighi & Hsu (36).

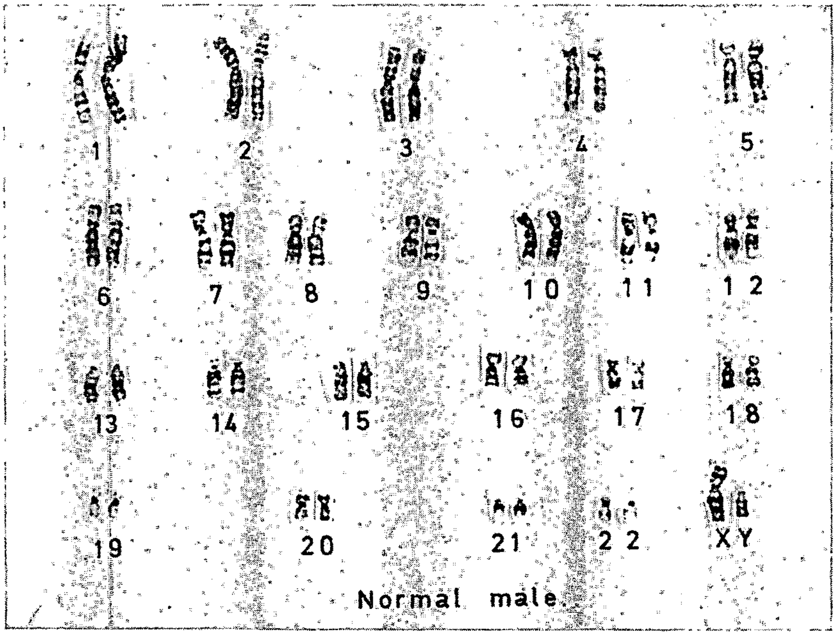

FIGURE 4. Karyotype of normal male stained with Giemsa after trypsin treatment by method described in text.

have already proven useful in defining structural abnormalities of human chromosomes and in improving the accuracy of genetic counseling (58, 60). These techniques have also shown characteristic patterns in other species (61, 62).

*Other techniques.*—Additional techniques for specific staining patterns of chromosomes have used slides followed by staining with Giemsa treated at 80°C, which results in a reversal (R-banding) of the patterns obtained with G-banding (63); fluorescent staining with acridine orange (64); and 24-hr treatment of the chromosomes before fixation with isonicotinic or para-aminosalicylic acid (65).

## Discussion

The various techniques used for identification of chromosomes have obviously provided a new tool with which to characterize chromosomal abnormalities in individuals or tumor cells with a high degree of accuracy. Reciprocal translocations can now not only be correctly described but even detected when the two exchanged pieces are of equal size (58), a situation which was not discernible with previous techniques. Far greater accuracy of genetic counseling will obviously result. In addition, these techniques are providing us with rapidly increasing information about the basic structure of chromosomes. For example, the finding that the secondary constrictions in chromosomes 1, 9, and 16 stain with the C-banding technique, do not stain with the Q-banding technique, and stain differentially with the G-banding (1 and 16 positive, 9 negative) tells us that there must be different species of DNA-containing short-chain repetitive units. This has already been confirmed by the exclusive staining of the secondary constriction of chromosome 9 by Giemsa at pH 11 (66), and by the findings that one fraction of human satellite DNA separated by differential centrifugation is in the secondary constriction of chromosomes 1 and 16 (67), while another class is in the secondary constriction of chromosome 9 (68). Until now no differences have been found in the staining patterns of cells from different tissues, at least by the Q-banding technique (69). The fact that proteolytic treatment results in specific banding patterns for all chromosomes implies that either some sections of DNA are covered by a different class of protein or that the DNA-protein complex in some areas is more susceptible to proteolytic activity. The observation that trypsin can enhance the template activity of isolated lymphocyte nuclei for new RNA synthesis (70) may shed light on this problem. It would appear that the staining patterns are related to the presence of various species of heterochromatin or genetically inactive DNA, generally considered to be late replicating, but the definition and characterization of this has long been an unresolved research question (71). It is, however, apparent that not all classes of heterochromatin stain differentially with these methods, since the late-replicating X in females, which consists of so-called facultative heterochromatin, stains identically to the other, genetically active X.

At a recent conference (72) a new nomenclature system was devised so that individual regions and bands of chromosomes can be accurately designated.

Essentially, the short and long arms are called *p* and *q*. Regions of each arm are numbered and divided by major landmarks, while individual bands are numbered within the regions. For example, the unstained band in the short arm of chromosome 6 (Figure 4) is referred to as *p*21, where *p* is the short arm, 2 is the second region from the centromere, and 1 is the first band in that region.

In conclusion, the recent technical advances in cytogenetics have brought a new order of accuracy to chromosome identification, and will no doubt result in vast improvement of genetic diagnosis and counseling, chromosome mapping, and study of chromosomal structure and function.

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# RADIATION AND DRUG THERAPIES, AND LEUKEMIA

*7061*

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Radiation has been suspected of causing human leukemia for more than 50 years, though quantification of such parameters as dose and incidence of leukemia is still unachieved. It is only in the last ten years that certain drug therapies have come under similar suspicion. As will be evident from the discussion below, there are many gaps in our knowledge of a possible drug-leukemia relationship. However, it may be appropriate, even at this premature juncture, to set down what is known about this disturbing possibility, in the hope that future factual material may be attached to some preexisting framework and any appropriate physician cautions may be taken at the earliest possible time.

It is now well established that exposure of human populations to various forms of radiation (X ray, gamma ray, neutrons) is followed by an increased incidence of leukemia (1–3). Studies demonstrating this connection have of necessity been epidemiologic, largely retrospective, and often hampered by inexact dosimetry, inappropriate control groups, short and/or incomplete follow-up of the exposed populations, and other conceptual or practical difficulties. Because of these difficulties, it is not yet possible to answer clearly some of the crucial questions related to radiation leukemogenesis. What is the relative leukemogenic potential of the various forms of radiation? Is there a threshold dose below which radiation will not produce an increased incidence of leukemia? If there is a threshold dose, does it vary with whether the dose is delivered as a single exposure or in fractionated fashion? Does the threshold vary with where the dose is delivered, e.g. total body or bone marrow, or when the dose is delivered, i.e. in prenatal growth or adult periods? Is it important whether the dose is delivered to normal persons or to those with nonmalignant or malignant disease?

The extensive data obtained by the Atomic Bomb Casualty Commission (4–7) come closest to answering some of these questions, although these studies suffer from several of the previously mentioned difficulties. In summary, these studies have shown the following: (*a*) There is a progressively increasing risk of acute lymphocytic, acute myeloid, acute undifferentiated, and chronic myeloid, but not of chronic lymphocytic leukemia with a single exposure of about 50 rads or more. (*b*) Between 100 and 500 rads the dose response curve approaches linearity. (*c*) When the linear part of the curve is extrapolated to zero rads exposure it intercepts at or near the expected incidence of leukemia in the nonradiated population. (*d*) The increase in leukemia incidence begins after a latent period of about 12–18 months, peaks at about 5 years, and continues for at least 25 years. (*e*) The distribution of types of leukemia by age group is similar to that found in "spontaneous" disease. (*f*) At dose levels of 100 rads or more the increase in incidence approximates 1–2 cases per $10^6$ persons at risk per year per rad.

The studies of Court Brown, Doll & associates on 14,554 patients with ankylosing spondylitis treated by X-ray therapy between 1935 and 1954 show a marked excess in incidence of acute leukemia (almost ten fold), and a dose-response relation which is roughly linear below 1250 rads. Again, if the linear portion of the curve is extrapolated to zero rads, it intercepts at or near the expected leukemia rate (8–12).

Many other studies have been carried out attempting to show a relation between therapeutic or diagnostic exposure to radiation and increased incidence of leukemia in exposed populations. Results have often been conflicting and many of the studies suffered from some or all of the previously outlined difficulties. An increased incidence of malignant disease in children exposed pre- and postnatally to diagnostic X rays has been claimed (13, 14), denied (15–17), and extensively debated (18, 19). Simpson et al showed that children treated with X-ray therapy for thymic enlargement had a clearcut increased risk of leukemia and thyroid cancer (20). Unfortunately, a cohort of children with thymic enlargement not treated with X-ray therapy was not available as a control group. The crucial necessity of such a control group is shown by studies of the incidence of leukemia following treatment of hyperthyroidism. The striking finding here was that leukemia incidence was significantly increased over that expected in both the radiation-treated $^{131}$I and the surgically treated groups (21, 22). This suggests that the underlying disease, hyperthyroidism, is a more significant factor in leukemogenesis then the therapeutic modality. Two retrospective partially controlled studies have attempted to define the contribution of diagnostic and therapeutic X-ray exposure to the total leukemia experience of defined populations. Stewart et al concluded that about 8% of nonlymphocytic leukemias were related to diagnostic X-ray exposure and a further 3.6% to therapeutic X ray (23). Gunz & Atkinson showed that patients with acute and chronic myeloid leukemia had a radiation excess exposure of only about 3% over controls (24). They concluded that diagnostic procedures contributed to less than 1% of acute and chronic myeloid leukemias, if they are a factor at all. In controlled studies therapeutic radiation has been linked to a six- to tenfold increased leukemia rate in Hodgkin's disease (25–29) and in metropathia hemorrhagica (30). In contrast no increase in incidence of leukemia has yet been shown in a large study of women radiated for cancer of the cervix uteri (31). The need for carefully selected control groups in interpreting such data cannot be overemphasized. The careful study by Modan & Lilienfeld (32) of the possible contribution of radiotherapy to the development of acute leukemia in polycythemia vera, polycythemia of uncertain origin, and secondary polycythemia showed that 11% of polycythemia vera patients treated with $^{32}$P and 8.9% of the polycythemia vera patients treated with X-ray therapy developed acute leukemia, compared to less than 1% of patients who received no radiotherapy. Similar results were found in the group with polycythemia of uncertain origin. Suggestive but not conclusive evidence of increased leukemia rates in patients with secondary polycythemia treated with radiation was also found. The increase in leukemia rate was to some extent dose-related, but was not necessarily related to increased survivorship. These conclusions have been, in essence, confirmed in later studies (33–35). However, caution must be used in interpreting studies comparing patients with polycythemia vera treated by cytotoxic agents with those treated with radiation in view of the possible leukemogenic potential of the former agents discussed below.

Because the available studies of patients treated with $^{32}P$ or X-ray therapy for chronic lymphocytic leukemia are uncontrolled (36–40), they cannot be used conclusively to support or deny the role of radiation in the "acute leukemic" (blastic) termination of 2 to 10% of patients in these series. However, in 1269 cases of "lymphosarcoma" in which 7.6% developed "leukemic transformation," only 0.8% (7.2% children, 0.4% adults) of these patients terminated in acute leukemia (41).

Recently cytotoxic agents have had clinical trials in a variety of diseases thought to be initiated or perpetuated by altered immune function (42–50). Some of these studies have shown encouraging therapeutic results, and it is to be anticipated that cytotoxic drugs will soon be widely employed in the treatment of certain nonneoplastic diseases. The direct, dose-related effects of these agents on rapidly dividing cells are well known (51) and are weighed in the therapeutic decision to use them. However, unexpected delayed effects from these agents are largely unknown because previously such agents were used in the treatment of patients with intrinsically short survival times (i.e. widespread malignant tumor).

Because of the potentially widespread, long-term use of cytotoxic agents in nonmalignant disease, reports of possible leukemogenic effects of these agents have been received with extraordinary interest and concern. In 1966 Nordenson reviewed 310 cases of myelomatosis (52). Two cases had died of myeloblastic leukemia (0.6%) and five had had concurrent lymphocytic leukemia (1.6%). No details of the therapy of these cases were published. In 1967, in a review of six cases of extraosseous lesions in plasma cell myeloma Edwards & Zawadzki (53) reported one patient who died of acute myeloid leukemia ten years after the diagnosis of multiple myeloma was made. This patient had received radiotherapy as well as urethane and cyclophosphamide.

During 1970–1971 three major reports were published linking melphalan therapy in multiple myeloma to acute leukemia. Kyle et al (54) reported acute myelomonocytic leukemia occurring in three patients with multiple myeloma and one patient with systemic amyloidosis seen in their clinic. They calculated that this represented a 200-times increased leukemia rate over that expected. Two of the patients had been treated with X-ray therapy. All had been treated with melphalan for periods varying from 30–57 months. This report also notes a case of Dr. Paul Carbone's in which a patient with multiple myeloma treated with melphalan died of acute leukemia. Details of this case are not given. Andersen & Videbaek (55) reported three definite cases and one probable (Case 3) case of "stem cell leukemia" occurring in 19 new cases of myelomatosis seen in their hematology clinic in four years (20%). Two of these cases received X-ray therapy, and all received cytotoxic drug therapy (2 melphalan alone, 1 melphalan and cyclophosphamide, 1 cyclophosphamide alone). In addition three patients received prednisone. Osserman (56) reported three cases of monocytic and monomyelocytic leukemia as a late complication in plasma cell myeloma. One case received radiotherapy, urethane, and melphalan; one melphalan and androgens; and one melphalan, cyclophosphamide, and androgens. The duration of multiple myeloma before the development of leukemia varied between four and seven years.

Three thoughtful editorials (57–59) have reviewed these cases and speculated on their significance. In one editorial, Holland added five patients with multiple myeloma treated with melphalan for 14 to 72 months who had died with acute leukemia. However, all five had also received X-ray therapy. Several single case reports note the development of acute leukemia in patients with a variety of malignant diseases treated with cytotoxic agents and no X-ray therapy (60–63, 40).

In addition to these published reports, the present authors personally have seen three patients with lymphomas treated with cytotoxic agents and no X-ray therapy for four to eight years who developed acute myeloid leukemia (64).

The significance of these data is difficult to evaluate. All patients reported at the time of this writing who developed acute myeloid leukemia while on cytotoxic agents were being treated for underlying malignant disease. The necessary control populations (patients with similar disease, untreated, with the same longevity as the studied groups) are not available.

On occasion other therapeutic agents have been associated with the development of acute leukemia. In 1960 Bean (65) reported the occurrence of three cases of myeloid and three cases of lymphocytic leukemia in an unspecified population receiving phenylbutazone. The relationship of phenylbutazone administration to the development of leukemia was considerably more convincing in the myeloid leukemias than in the lymphocytic leukemias. In 1965 Dougan & Woodliff (66) reviewed the literature on the association of phenylbutazone and leukemia and presented a retrospective study in which they found that five of 55 (9%) registered patients with acute leukemia had received phenylbutazone, but had no known exposure to radiation or other known leukemogen before the development of leukemia. Only five of 417 (1.2%) registered patients with other malignant disorders were found to have received phenylbutazone before onset of the malignant disorder. In contrast, Fraumeni (67) performed a follow-up survey of 24 persons who had phenylbutazone-induced bone marrow depression and found no leukemia developing subsequently. However, his assumption that phenylbutazone-induced leukemia would be expected to follow only phenylbutazone-induced bone marrow depression may not be valid. Also the number of patients followed and the length of follow-up would probably be inadequate to detect any but the most gross increase in leukemia rate. Until a defined population receiving phenylbutazone and appropriate controls are studied, no firm conclusion on the leukemogenic potential of this drug can be reached. A similar situation exists with regard to the possible association of chloramphenicol administration and the subsequent development of acute leukemia (68, 69).

At the present time there is controversy about whether androgen administration to patients with aplastic anemia may cause or facilitate the development of acute myeloid leukemia (70–72). Because androgens alter the natural history of aplastic anemia and because the nature of the alteration is unclear, it is unlikely that any true estimate of the leukomogenic potential of androgens in aplastic anemia will be definable.

For the sake of completeness, it should be noted that chronic exposure to benzene, which is not a diagnostic or therapeutic agent, has been rather strongly associated with the development of acute myeloid and acute monocytic leukemia (73, 74). A possible mechanism was investigated by Tough & Court Brown who found increased chromosome aberrations in persons exposed to benzene as compared with suitable controls (75).

In contrast to radiation, cytotoxic agents, phenylbutazone, chloramphenicol, and benzene, which have been associated with myeloid leukemia, diphenylhydantoin, and renal transplantation have recently been associated with increased risk of lymphoreticular malignancy. The evidence that diphenylhydantoin can produce lymphadenopathy which histologically resembles lymphoma and which disappears on withdrawal of the drug (pseudolymphoma) is clear (76). That on occasion persons apparently recovered from diphenylhydantoin-induced pseudolymphoma may later develop true malignant lymphoma has also been documented (77, 78). Data on the exact risk of developing pseudolymphoma or true lymphoma in persons taking diphenylhydantoin are not yet available (79). Indeed, it cannot be unequivocally stated that diphenylhydantoin produces lymphoid malignancy or even that pseudolymphomas are pathogenetically related to the later development of true lymphoid malignancy. In contrast, the evidence associating renal transplantation (and the necessary use of cytotoxic immunosuppressive therapy and occasional X-ray therapy to prevent or treat rejection) and a markedly increased risk of developing lymphoid malignancy is convincing. In a review of lymphoma as a complication of allotransplantation in man, Pierce et al (80) report three cases of reticulum cell sarcoma in 151 transplant recipients. The patients were followed for a total of 438 patient-years yielding a lymphoma incidence of 0.7% per year, which is more than 100 times greater than the experience in the general population. The latter review of the available data on all renal transplant recipients yielded a calculated annual lymphoma mortality rate of 0.4%. It is noteworthy that many of the lymphomas developing in renal transplant recipients appear to be primary intracerebral disease, which is rarely seen in the general population of patients with lymphoma (81). An increased incidence of malignancies other than lymphomas is also found (82, 83). Penn et al (82) found a 6% occurrence of de novo malignant tumors in a group of transplant recipients followed between 18 months and 8 years after transplantation. This is to be compared with an expected rate of 0.058% per year. It has also been shown that renal transplant patients can support the growth of an accidently transplanted malignant tumor (84). That one such patient was able to reject a transplanted tumor after appropriate measures were taken is of great theoretical interest.

## DISCUSSION

There is clearly an association in humans between radiation therapy or certain drug therapies and the subsequent development of leukemia. Because of inherent problems in study design and the great difficulties of obtaining true control groups, it is not yet possible to quantify the risks of these various therapies. The most practical approach to obtaining such data will involve setting up central registries to study the end results of patient populations receiving these therapies for various diseases. Multivariate analysis of a large body of such data might well allow at least semiquantitative estimates of the leukemogenic potential of radiation therapy and certain drug therapies utilized in these various disease states.

Perhaps because the possibility of inducing a lethal disease with therapy violates the first principle of medical care, there has been an extraordinary amount of interest, concern, and speculation fostered by recent reports of therapy-related leukemia. Some of the proposed mechanisms for the induction of such cases of leukemia are discussed below.

(*a*) Chromosome breakage (somatic mutation): It is known that children with Bloom's syndrome and Fanconi's anemia have a tendency to chromosome breakage and also have an increased risk of developing leukemia and lymphoma (85). It is also well known that radiation and cytotoxic drugs can produce chromosome breaks (86). Therefore, it is postulated that the chromosome breakage produced by radiation or drug therapy may in some way produce a mutant malignant cell line.

(*b*) Activation of a latent leukemogenic agent (virus?): Kaplan (87) has reviewed the data on radiation leukemogenesis in mice. The results are not compatible with somatic mutation-producing leukemia. They do strongly suggest that radiation of mice in some way activates a transmissible agent (latent virus?) which itself is leukemogenic. There is no convincing evidence, at present, that this mechanism is applicable to human leukemogenesis.

(*c*) Immunosuppression: Patterson et al (88) have suggested that the cell-mediated immune response constitutes a surveillance system capable of identifying and destroying mutant cells. Suppression of this response might allow a mutant malignant clone of cells to grow. Since both radiation and cytotoxic drugs are immunosuppressive, it has been reasoned that this is the mechanism of leukemogenesis by these agents. Law (89) has reviewed the impressive animal data and Fahey (90) has reviewed some of the human evidence supporting the likelihood of this mechanism. Attempts to explain the high incidence of lymphoreticular neoplasia in kidney homograft recipients have centered around this mechanism. It has been suggested that continuous stimulation of the host's depressed immune system by the graft predisposes to the lymphoreticular malignancy which may be found (80). Alternatively, transplantation of foreign lymphocytes (with the graft or in transfused blood) in the immunosuppressed host may allow these cells to give rise to malignant tumors (91).

(*d*) Marrow aplasia: Both radiation and cytotoxic agents can produce bone marrow aplasia. It is possible that a regenerating marrow, under great stimulation to produce, may allow mutant-malignant cells to proliferate and produce disease.

It is worth noting that these postulated mechanisms are not mutually exclusive. For example, immunosuppression may allow activation of a latent leukemogenic virus which is able to multiply only in cells damaged by cytotoxic drugs. The discovery of the actual mechanisms involved in human therapy-related leukemia awaits further work.

## SUMMARY

The data associating accidental or therapeutic radiation and certain drug therapies with leukemia have been reviewed. Quantitative estimates of the leukemogenic potential of these therapies in various disease states are not yet available. Such estimates will require data from painstakingly maintained registries of persons receiving such therapies for various disease states, as well as data on suitable control subjects. Several plausible mechanisms explaining leukemogenesis by these agents have been suggested. It is not yet known which, if any, of these mechanisms apply to human disease.

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# URETHROVESICAL DYSFUNCTION AND INFECTION *7062*

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Recurrent infection of the lower urinary tract in women (bacterial cystitis) results from an instability between factors maintaining normal bacterial flora and those promoting changes in type and distribution of the flora.

It has been postulated (1) that if all urethral defenses are operating, fecal flora is restricted to the perianal area, but as natural barriers are breached, the perineum, urethra, and bladder are successively colonized and infection becomes established.

Important, then, are 1. the source of the pathogenic bacteria, 2. local factors permitting perineal and urethral colonization by pathogens and the effects of intrinsic defense mechanisms, and 3. the hydrodynamic mechanisms for movement of bacteria into the bladder and for their elimination.

## Source of Pathogenic Bacteria

The bowel serves as reservoir for urinary pathogens. In most hospitalized or domiciliary patients, the specific strain of *E. coli* causing the infection can be found in the feces (2, 3).

The normal female urethra contains few pathogens. Cox (4), using an adaptation of Helmholz's everting cot (5), obtained specimens from 52 normal female urethras and found pathogenic bacteria (usually *E. coli*) in only 2 to 9%. More recent studies by Stamey's group (6) showed *E. coli* in the vaginal vestibule or urethra in 13% of 30 normal premenopausal women. Moreover, the absolute number of bacteria is low and they are distributed in the distal urethra (4). In women with recurrent infection, more pathogenic bacteria are found at higher levels and colonization of the distal urethra precedes clinical bladder infection. Cox, Lacey & Hinman (7), by intermittently culturing the urethral flora in patients known to have recurrent cystitis, were able to detect a type-specific organism in the urethra at a time when bladder urine was sterile and the patient asymptomatic, only later to recover the identical organism from the bladder urine during a clinical attack.

Stamey (6) confirmed that colonization of the "introitus" by enterobacteria precedes each episode of bacteriuria. Conversely, it has been shown that topical application of antibacterial agents to the vulva reduces the incidence of infection in women susceptible to recurrences (8).

The female urethra may be infected without vesical bacteriuria. It is probable that a period of urethritis precedes overt cystitis. Moore & Hira (9) found that 82.4% of patients with lower urinary tract symptoms did not have bacterial cystitis, but had high numbers of inflammatory cells in the first part of the voided specimen compared to normal women. They concluded that involvement of the periurethral glands ("female prostatitis") was responsible. Others (10, 11) have made similar observations.

## FACTORS LIMITING PERINEAL AND URETHRAL COLONIZATION

Why the vestible and urethral meatus become pathologically colonized is unclear. Colonization has not been correlated with pH, hormonal status, or the presence of mycoplasma or anaerobic bacteria (6). Rather, the explanation lies in the malfunction of the urethral defenses.

In early studies (12), we descibed two local defense mechanisms: 1. intrinsic defenses involved with secretions and mucosal activities and 2. those concerned with mechanical washout brought about by periodic voiding.

Intrinsic defenses may include surface antibacterial activity, functional microplicae, trapping mechanisms, hormonal effects, local secretions, and immune and inflammatory responses. They are important in limiting colonization of the urethra and the ascent of bacteria. Most of the information comes from experiments on bladder mucosa, but there is reason to believe that bladder and urethra have properties in common.

Cobbs & Kaye (13) demonstrated antibacterial activity in the bladder of rats, and felt that infiltrating leukocytes were responsible. Norden, Green & Kass (14) found antibacterial activity in rat and guinea pig bladders to be related to the volume of urine relative to the mucosal surface area. Gillenwater and associates (15) repeated these experiments in nonvoiding rats, using radioactively tagged *E. coli.* They found different degrees of antibacterial activity with various strains of *E. coli* and also noted a relationship between volume and surface area of the bladder. Bacteria were killed as soon as 30 min after inoculation. The rate was not affected by leukopenia. Hence they concluded that phagocytosis by leukocytes was not likely to be the mechanism of killing in these experiments but rather some specific but weak bactericidal secretion of the mucosa. However, the urinary tract mucosa and submucosa show a very active initial inflammatory response to the presence of pathogenic bacteria, with infiltration of the submucosa by both polymorphonuclear leukocytes and lymphocytes. Leukocytes are also found between the mucosal cells as though the former were entering or leaving the lumen of the bladder (16).

In experimental infection in animals the cell surfaces are seen—by scanning electron microscopy—to become much more irregular and to show surface collections of bacteria (17). Such fixation of bacteria might well decrease the likelihood of washout during micturition but might also hold the bacteria to allow eradication by leukocytes and immune reactions.

An influence of estrogens on the urethral mucosa has been known for some time (18) and local application of estrogens is accepted treatment for urethritis and resultant cystitis in postmenopausal women. The fact that newborns have greater urethral caliber than infants (19) and a rapid increase in caliber occurs at puberty (20) also suggests a hormonal effect on the urethra. However, in premenopausal women, Fair and associates (21) found no difference between the cytology observed in Papanicolaou smears from the vestibules of women with recurrent infection and those from normal volunteers.

Although male prostatic secretions are antibacterial (22), so far no evidence is available that glandular secretions from a "female prostate" would also be antibacterial.

The immune globulin IgA has been shown to exist in relatively higher concentrations than other immune globulins on the bladder mucosa (23) as on other mucosal surfaces and may (by analogy with its demonstrated effects in the respiratory and gastrointestinal tracts) play a role in local immunity. The normal microbial flora may be a major stimulus for the production of IgA (24). Tourville (25) believes it likely that the immune globulins with antibacterial activity against potentially invasive bacteria play an important part in the defense of mucosal surfaces against colonization by pathogenic organisms. In fact, IgA has been found around bacteria obtained from the urinary tract. However, Hand and associates (26) were unable to detect an increase in IgA after experimental infection, but found a great rise in IgG. Their conclusion was the same, that a significant local immune response occurs. Drach (16) was able to show in the mucosa of bladder specimens obtained from 4 of 13 patients with urinary tract infection the presence of immunofluorescence to type-specific *E. coli* antigen. Thus, reactions to antigens do occur, at least at the luminal surface of the bladder.

Alterations in intrinsic defenses have been studied experimentally in the female dog by performing episiotomies to expose the urethral meatus (27). Bacterial counts rise at all levels in the urethra. In pregnant women, urethral bacteria decrease late in pregnancy (28). Whether this is due to change in hormonal environment or to change in coital activity has not been determined.

Change in immune mechanisms may play a role. If female dogs are given an immunosuppressive drug (azathioprine), little change occurs in the flora of the urethra. Similarly, if the urethra is vigorously instrumented, the flora remains the same. But if the immunized dog is instrumented, the flora increases dramatically (29). Thus, the defenses against injury are reduced by immunosuppression.

## Hydrodynamic Mechanisms for Movement of Bacteria into the Bladder

Micturition provides periodic flushing of the urethra with sterile urine. Such washout is incomplete because of certain macroscopic and microscopic characteristics of the urethral wall. Urethral macrocontour is grossly irregular and the rate of urinary flow is relatively low (with a Reynold's number under 2000); thus, flow is turbulent and standing vortices are likely. Observation of urethral mucosal microcontour under the scanning electron microscope (30) reveals pits and folds of the cell surface (31) that may catch bacteria to be held on the surface by mucus or other substances (32).

Alterations in flow may occur in pathologic states. Washout becomes less effective if the urethra is narrowed distally, because of sequestration of bacteria in back-eddies. Experiments in female dogs (29) have shown that when urethral meatal stenosis is present, higher colony counts exist in the urethra, and that these are reduced by instrumental dilatation of the urethral meatus.

Removal of flow altogether by urinary diversion permits increased numbers and more proximal distribution of bacteria, as shown in other experiments on female dogs (29). Thus, periodic washout appears to be an essential factor in maintenance of normal urethral flora. At a microscopic level, washout appears to be enhanced by stretching the surface of the cells (30). The very irregular surface seen by scanning electron microscopy during infection (18) would reduce washout. Moreover, bacteria are seen to adhere to the surface (32), and hence are resistant to displacement by flow.

Movement of bacteria from urethra to bladder can occur between voidings if they lie above the midurethral high pressure zone. Experiments in dogs showed that bacteria placed distally in the urethra rarely moved up into the bladder, but, if placed at the high pressure zone slightly distal to midurethra, they were moved up and could be recovered in the bladder aspirate (33). However, recovery of bacteria from aspirated bladder urine after digital manipulation of the urethra in 9 of 24 women (34) demonstrates the ease with which retrograde movement of the normal urethral flora can occur. Coitus involves similar, more vigorous penipulation.

During micturition, if the stream is suddenly stopped voluntarily (or reflexly because of irritation) the proximal two thirds of the urethra become continuous with the bladder (35) so that bacteria mobilized from the walls by the turbulent flow and standing vortices may gain access to the bladder (36). Experimental evidence for such backflow was published by Corriere and associates (37) who, after inoculation of the urethra with radioactive particles, found no contamination of the bladder if it was emptied passively by suprapubic pressure but found 7 of 20 bladders contaminated if the animal voided spontaneously.

The hesitant voider, described by Lyon & Tanagho (38), and Tanagho and associates (39), suffers irritation from distal urethral stenosis and so reflexly interrupts the stream. With each cutoff, bacteria may gain access to the bladder if they are distributed above the most distal urethra.

Observations on women with sterile bladder urine who required retention catheterization after renal transplantation (40) showed that if bacteriuria developed, it was often due to the same species that was cultured from the catheter upon its removal. The interpretation is that the urethra contaminated by the presence of the catheter was unable to clear itself and so allowed bacteria to ascend and become established in the bladder.

Although bacteria may reach the bladder, infection (bacteriuria) does not result unless the bladder emptying mechanism is faulty and residual urine exists (41). Since urethritis precedes cystitis (42, 43), the urethral inflammation when severe produces enough dysfunction to both disturb urethral washout and inhibit complete vesical emptying.

## SUMMARY

An essentially nonpathogenic flora resides in the normal distal urethra. Alterations in the surface defenses of the perineum and the meatus permit colonization of the urethra with pathogenic bacteria of fecal origin. Anatomic abnormalities and disturbances of normal micturition may allow retrograde movement of bacteria to the bladder, where incomplete emptying fosters the establishment of bacteriuria.

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# BEHAVIORAL ASPECTS OF CHROMOSOMAL DISORDERS

7063

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Chromosomal abnormalities occur in approximately one out of every 200 liveborn children; about half involve the autosomes and half the sex chromosomes (1). In the USA alone, 18,000 to 20,000 children are born each year with a chromosomal disorder; at any given time over 1,000,000 individuals may be carriers of these abnormalities (2). The possibility that these anomalies contribute to intellectual deficits, antisocial behavior, and psychiatric problems has evoked considerable legal, medical, and social interest. The state of knowledge concerning the behavioral consequences of the major chromosomal disorders is considered here. For each, the major findings regarding cognitive defects, personality disorders, and psychopathy are assessed, and methodological issues and future research needs are discussed. Several reviews have recently appeared (1–5).

## SPECIFIC DISORDERS

*45,X females.*—The syndromes of ovarian dysgenesis comprise three major clinical entities: Turner's syndrome, Ovarian Dysgenesis, and Pure Gonadal Dysgenesis (6). Only the first two groups are considered here. In about 51% of cases with dysgenetic ovaries, a 45,X sex chromatin-negative karyotype is detected. In 18%, mosaicism, generally 45,X/46,XX, is found, and about 25% carry an X chromosome abnormal in structure. In most studies concerning the behavioral effects associated with the syndromes of ovarian dysgenesis, Turner's syndrome and Ovarian Dysgenesis are considered together as Turner's syndrome (TS). A comprehensive review of this literature may be found in Garron & Vander Stoep (7). Among newborn females, 45,X females occur at rates ranging between 0.0 and 0.6 per 1,000 (8, 9); the mean of several studiesis 0.2 per 1,000 (8).

Early reports suggested an association between TS and mental subnormality (7, 10); however, surveys of institutions for the mentally subnormal revealed no marked increase in the numbers of these patients above that expected on the basis of the newborn incidence (10, 11). In 11 different studies involving 8,882 females in hospitals for the mentally subnormal, only 5 chromatin-negative women (0.6 per 1,000) were found (12). However, among mentally defective girls enrolled in special classes in Japan, chromatin-negative females occur at a rate

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[1] Supported by MH 8304.
[2] Supported by MH 16026.

of 1.3 per 1,000 (9), and the distribution of IQ scores of TS patients seen in endocrine and fertility clinics suggests that these individuals are overrepresented in the lower IQ range (13). Thus, a risk for mental retardation associated with TS cannot be ruled out at the present time.

Money and co-workers studied a group of 38 TS females and found a wide distribution of IQ scores ranging from below 70 to above 130 with a mean of 96 (14). No significant differences were found between the IQ scores of 45,X mosaic, translocation, and deletion TS individuals. Also, no evidence of an association between IQ and the presence or absence of neck webbing was found (10). However, recent data cited by Polani (1) suggest that mosaics tend to have relatively higher IQ scores than 45,X females.

Several investigators have reported a marked discrepancy between the mean performance and verbal components of the Wechsler IQ test in TS patients (14–16). Money found that the mean performance IQ of 37 TS females was 87.6 and the mean verbal score was 104.6; the difference was statistically significant (14). This discrepancy was not found in a group of Klinefelter's patients (14), in 5 TS patients under 13 years of age (13), nor in 15 45,X females diagnosed with Ovarian Dysgenesis (1). The impairment in performance scores may account for the apparent increased incidence of mild retardation among TS individuals (7).

Money and co-workers have interpreted the lowered perceptual organization scores found among TS subjects as representing a cognitive defect, namely a dysgnosia for space-form perception (space-form blindness). Workers in Boston (15) and Leiden (13) have independently confirmed the presence of spatial deficiencies among TS subjects. Further support for the existence of some kind of space-form perceptual difficulty among TS individuals was found in their lowered scores on various design and drawing tests (13, 16, 18). Space-form blindness occurs in about 75% of cases and with varying degrees of severity (17).

A deficit in right-left spatial orientation limited to extrapersonal space has also been found among TS subjects, and the presence of a neurological defect of the parietal lobes has been suggested (19).

Garron & Vander Stoep (7) suggest that the space-form deficits associated with TS occur because these individuals are poor at tasks which are primarily nonverbal, but are unimpaired on verbal tasks. These authors criticize the space-form blindness notion on several grounds: 1. the distinction between personal and extrapersonal space may be a distinction between less and more difficult tasks; 2. although numerical and spatial abilities may be related, no marked evidence of dyscalculia has been found (13, 19); and 3. since reading ability appears to depend on the perception of form and orientation, dyslexia would be expected, but this too has not been found (20).

Considerable attention has been paid to the gender orientation of Turner's patients. Interviews (21, 22) and assessment by the Rorschach (15, 23), TAT (15), DAP (15, 24), MMPI (25), GZTS (25), and other tests all suggest that these individuals are female in their gender orientation. Erhardt et al (24) compared 15 diagnosed TS cases to 15 chromosomally normal controls matched by age, race, IQ, and father's occupational level, and found that the former were as feminine in their childhood sexual activities and interests in marriage, romance, and maternalism as the controls. They also had a somewhat greater interest in jewelry and cosmetics and were more passive with respect to outdoor activities and childhood fights than the control group.

Other characteristics which have been applied to TS patients include reduced libido in adulthood (26); low levels of enthusiasm, energy, and impulsiveness (21, 22, 25); and immaturity, sensitivity, and passivity (27). No consistent relationship between the presence of psychopathy and physical stigmata has been found (17). When psychopathology was present, it was generally associated with disturbed intrafamilial relationships. It has been suggested " . . . that the genetic defect that produces the adverse symptoms and stigmata of Turner's syndrome produces also a beneficial effect, so far as the personality is concerned" (17).

*47,XXX females.*—47,XXX females occur among newborn females at a rate ranging from 0.3 to 1.4 per 1,000 with a mean of about 1.1 per 1,000 (8). 46,XX/47,XXX is the mosaicism most often encountered and is generally included in the cytological and psychological assessments of XXX females. In their review of the literature, Barr et al (28) compiled 70 cases out of 16,352 individuals tested in institutions and special schools for the mentally retarded, a rate of 4.3 per 1,000. It has been suggested that mental retardation is a characteristic feature of XXX females (1). Several investigators, however, have shown that factors other than the supernumerary X chromosomes may be responsible for the mental subnormality frequently found. These include prematurity (29), birth complications (30), the presence of athyreotic cretinism (30), and a family history of consanguinity (30), mental retardation (30, 31), and congenital anomalies (32). Also, in cases detected outside of institutions for the mentally subnormal, average or above-average intelligence has been described (32–36). Family studies are needed before a firm conclusion regarding the association between an extra X chromosome and mental retardation can be drawn.

In hospitals for the mentally ill, XXX individuals appear to occur at a rate of 2.7 per 1,000 females (28); Vartanyan & Gindills (37) suggest that among schizophrenic women the rate may be 3.5 per 1,000. Combining the compilation of Barr et al (28) with several recent reports (37–39), the number of XXX and related mosaic females found in mental institutions is 42/15,993 or 2.6 per 1,000. Examination of these data, however, reveals that a substantial fraction of the cases are of advanced age. For example, in 28 cases for which the information was available, the median age was 59 years: 46% were 60 or more years of age, only 21.4% of the cases were less than 45 years of age, and only 2 cases were 35 years or less. These findings are of interest in light of reports showing a trend towards an increased incidence of aneuploid chromosomal counts in females over 35 years of age (40, 41).

*47,XXY males (Klinefelter's syndrome).*—The incidence of sex chromatin-positive males among the newborn is approximately 2.2 per 1,000 (1); of these about two thirds are 47,XXY and one third mosaics, mostly 46,XY/47,XXY. In newborn surveys, XXY males occur at rates ranging between 0.8 and 2.3 per 1,000 with a mean of about 1.2 per 1,000 (8). 48,XXXY and 48,XXYY males tend to show physical features similar to XXY individuals (12).

Early studies of males with a diagnosis of Klinefelter's syndrome (KS) suggested that intellectual deficits were present (42, 43). IQ testing of XXY males has been carried out in mental hospitals, institutions for the mentally subnormal, penal institutions, and in fertility and other outpatient clinics (44). In general, IQ scores are found to vary widely. In hospitals for the mentally subnormal, chromatin-positive males occur at a rate of about 9.4 per 1,000 (12). In outpatient groups, average and above-average scores are found (14, 45–47). Mental retardation and psychopathology may be present among the relatives of some retarded KS males (43).

XY/XXY individuals appear to be less severely affected physically and physiologically, as well as intellectually, than nonmosaic KS cases (12).

A broad range of personality characteristics have been associated with KS including alcoholism, criminality, exhibitionism, homosexuality, transvestism, pedophilia, and psychosis. In a comparison of 34 XXY and 16 hypogonadal XY age-matched men seen at a fertility clinic, blind ratings showed that the former had significantly more pathological symptoms (e.g. below-average school performance, little energy or unusually poor relations with parents or siblings, and previous history of mental illness) than the latter (48). The XXY men were also characterized by less well-developed body concepts, less freedom from distractability, and less psychological maturity and differentiation than the controls.

Data compiled by Maclean et al (49) suggest that chromatin-positive males are found in mental hospitals at a rate of about 5.4 per 1,000. Several investigators have found an association between KS and schizophrenia (37, 48, 50–52), but this apparent association may largely reflect a nonspecific influence of the extra X chromosome in males on the development of psychosis (49). No chromosomal disorders were found among the adult schizophrenics studied by Judd & Brandkamp (53).

Court Brown (54) found that among XXY males in institutions for mental defectives, 30% had been admitted for such antisocial acts as larceny, arson, and indecent exposure. He suggested that an abnormal sex chromosome complement may predispose towards delinquent behavior. In subsequent research, support (55) and no support (56) for this position was found. Chromosomal surveys in penal and special security institutions suggest that XXY males may be found at rates higher than that expected on the basis of the newborn incidence. Differences in methods of ascertainment, age range, racial composition, and other factors limit the conclusions which can be drawn from this apparent increase.

Although instances of homosexuality have been reported among KS patients, sex chromatin and chromosomal studies of homosexual populations have failed to yield large numbers of XXY males (57, 58). Studies of small samples of transvestites and transsexualists failed to show any cases with abnormal chromosomal constitutions (59–61).

Although the physical abnormalities associated with KS generally become apparent at puberty, several investigators have suggested that behavioral difficulties become apparent prepubertally (43, 62, 63), often once school is started. Learning disorders, stuttering, aggressiveness, antisocial acts, and difficulties in relating to other children have been reported (47, 62–64). These findings suggest that behavioral disorders similar to those seen in minimal brain damaged children may appear in KS children, placing them at greater than average risk for behavioral dysfunction later on.

*47,XYY males.*—In chromosomal surveys among newborn males, XYY males occur at rates ranging from 1.4 to 3.8 per 1,000; the mean is about 1.5 per 1,000 (8). Since most cases tested were institutionalized, no conclusions can be drawn regarding the intellectual capacity of these men. In some cases, average and above-average IQ scores have been observed (65), and when compared to matched XY controls no differences are generally found (66, 67).

Considerable attention has been focussed on the possible predisposition to hyperaggressivity and criminality among XYY males. Among 315 men in a state hospital in Scotland, 9 cases were found—nearly 3% of the group (68, 69); on average these cases were six inches taller than the XY inmates. It was claimed that criminal activity began substantially earlier among the XYY cases and that there was no history of criminality among their relatives (69). Kessler & Moos (70) provide a critique of these data. Subsequent studies have shown no remarkable differences between XYY and XY criminals with respect to age at first conviction (71, 72), and it is now clear that many institutionalized XYY cases do come from families that show evidence of interpersonal or psychosocial disturbance (73–75).

Institutionalized XYY men have been described as impulsive, unrealistic, loners and drifters, disruptive, and as underachievers in their school histories (76); however, on personality tests administered to XYY males and matched fellow inmates, no significant differences have emerged (66, 67).

The association between the XYY karyotype and unusual impulsivity and aggressivity has been questioned (70). Attempts to detect XYY men on the basis of high psychopathy scores on a personality test (77) or among unusually aggressive, dangerous, or violent convicts (78, 79) have not been successful.

XYY males are sometimes found in penal institutions at rates 20 or more times higher than that expected on the basis of their newborn incidence (80). It is not clear whether these findings represent sampling bias, random sampling variation, or a true predisposition towards institutionalization as a consequence of having an extra Y chromosome. In the most extensive and systematic survey thus far reported, involving 2,538 inmates in a variety of prisons and corrective institutions in Scotland, no increase of XYY males above that expected on the basis of the newborn rates was found (81). If confirmed in other populations, this finding would suggest that there is no increased risk for criminality among XYY males.

*Trisomy-G (Down's syndrome).*—The incidence of Down's syndrome (DS) among live births ranges from 0.6 to 1.4 per 1,000 with a mean of about 0.8 per

1,000 (8). About 94.5% of DS cases show the presence of an extra G-group chromosome, defined as number 21; these constitute the 21-trisomic group. The remainder largely involve D/G or G/G translocations and various mosaic types. Although the use of antibiotics has extended their life expectancy, a considerable proportion of DS cases die in the first year of life. In institutions, DS patients represent 10% (83) or more (2) of all cases.

Early studies of institutionalized DS patients showed that between 25 and 38% had IQ scores below 25, and 61 to 72% fell within the range of 25 to 49 (82). Noninstitutionalized DS subjects, however, achieve IQ scores within ranges of mild to moderate retardation (84). Compared to non-DS subjects of comparable intellectual levels, deficits appear to exist among DS subjects in stereognostic recognition, copying and reproducing geometrical designs, reaction times, verbal tasks, speech and language scales, sensory functions, and speed of perception (85). Dingman (86), however, found no differences in linguistic ability, hand-eye psychomotor coordination, perceptual speed, and figural reasoning in DS subjects matched to other retardates for mental age.

Dicks-Mireaux (87) administered the Gesell developmental scale to DS infants, all 21-trisomics, and to a group of 100 normal infants, 20 each at 16, 28, 40, 52, and 78 weeks of age. It was found that the DS subjects had a below-average developmental quotient (DQ) as early as 16 weeks of age, and in their longitudinal development showed a significantly slower rate of development and a progressive deterioration between the ages studied. The controls, on the other hand, conformed to the expected pattern of development.

Several investigators have pointed out that the IQ scores achieved by home-reared DS cases tend to be higher than those of institutionalized ones (88). Birch & Belmont (89) provide a discussion of the methodological problems inherent in the research in this area, and suggest that inadequate institutional practices may be responsible for the disturbances in intellectual and developmental progress of institutionalized cases.

Francis (90) found that institutionalized DS and non-DS children were more retarded in motor and visual activities than their home-reared counterparts of the same chronological age. When the institutionalized and home-reared DS subjects were equated for developmental levels, as many behavioral differences were apparent as when comparisons were made by chronological age. These findings suggest that the institutional environment was important in producing the developmental and behavioral retardation. Francis (91) reported that the incidence of object-oriented behaviors decreased and self-oriented behaviors such as rocking, diffuse movements, and posturing increased with age among institutionalized DS subjects. When an increased level of social contact was provided, the frequency of behaviors resembled that of the home-reared group of DS subjects.

A slightly higher mean IQ was found among translocation (92, 93) and among mosaic (94, 95) DS cases than among trisomics. Some studies show no IQ differences between mosaic and trisomics (93). With respect to the relationship between physical stigmata and IQ, positive, negative, and no correlations have been found. Baumeister & Williams (96) discuss this literature.

Attempts have been made to attribute a characteristic constellation of personality traits to DS subjects (97), but some workers have found no greater homogeneity of personality among DS cases than among other retardates of comparable mental age (84).

## METHODOLOGICAL AND CONCEPTUAL ISSUES

Two major methods have been used to establish an association between a chromosomal disorder and behavior: comparison of the incidence of the disorder among institutional groups to the incidence among the newborn, and comparison of groups with an abnormal chromosomal constitution to chromosomally normal controls. Each method has potential biases which may lead to spurious associations between a given karyotype and behavior. First, there is variation in newborn incidence rates of chromosomal disorders, partly due to the fact that the data are largely compiled from hospital births (12), which themselves are influenced by a number of socioeconomic, medical, and biological factors. Variations in practices governing the admission of women for confinement, regional and national differences in age at marriage, and preferred family size suggest that hospital births do not necessarily provide an unselected sample of the total liveborn population. Newborn incidences of chromosomal disorders are also affected by changing marriage patterns and fertility rates, regional differences, and multiple factors which modify the rates of chromosomal nondisjunction (98). Also, sex chromosome abnormalities may occur more frequently among lower socioeconomic-status families than among higher ones (99). Thus there are multiple factors which may influence newborn incidences of chromosomal abnormalities and which could bias the estimates of risk for behavioral dysfunction in adult groups. Differential postnatal mortality among carriers of certain abnormalities (e.g. 45,X females) relative to normals would tend to produce an underestimate of risks for behavioral disorder among adult groups.

Second, variations of incidence rates among institutionalized populations are as great or greater than variations among newborn populations. For example, the frequency of chromatin-positive males ranges from 4.4 to 19.8 per 1,000 in hospitals for the mentally subnormal; these rates may be related to the regulations governing admission and discharge policies (12). Some hospitals do not accept patients with severe behavioral problems whereas other hospitals specifically select for individuals who are mentally subnormal and who have severe behavioral disorders. Also, there are regional and local variations in the criteria by which individuals are labeled as educationally subnormal or behaviorally deviant.

Other factors which potentially bias the incidence of chromosomally abnormal individuals in institutions are height and marital status. More XYY individuals may be institutionalized because taller individuals tend to be arrested more often and/or because XYY individuals may lack the intelligence and facility to evade arrest (100). The possibility that they are infertile and thus less likely to be shown leniency in sentencing than fathers may account for the fact that some KS individuals may be institutionalized (4). A related problem is that the phenotypic characteristics of individuals with chromosomal abnormalities vary depending upon how and where they are ascertained. For example, XXY males referred to endocrinology clinics tend to show the highest frequencies of gynecomastia and female pubic hair and shave the least often, whereas males referred from subfertility clinics have masculine characteristics to an extent not shown by other XXY males (12). That sampling in institutions may identify individuals different from those identified in sampling from outpatient clinics is a well-known problem in human behavior genetics research and is discussed elsewhere (101) in relation to concordance rates in twin studies.

Another source of bias arises when aneuploidy tends to increase with advancing age, as for example among chromatin-positive females; institutional incidences might thus overestimate the risk for behavior dysfunction.

Third, in comparing groups with abnormal karyotypes with normal controls, other potential biases may be present. Some of these issues are discussed elsewhere (70). The effects of refusal to cooperate in a study may be important since refusal may be related to aggressive or rebellious tendencies (102). Also, variations in the characteristics used in matching abnormal and control groups can affect the results of comparisons in significant ways. The importance of matching for age is discussed by Court Brown (12).

Several factors may lead to spurious associations between an abnormal karyotype and behavior. For example, institutionalization per se may affect the intellectual and behavioral characteristics of individuals with abnormal karyotypes. This is indicated by the fact that IQ scores often decline a few points a year in young institutionalized children (103). Also, many of the psychological characteristics observed in association with certain karyotypes (e.g. TS) may be secondary consequences of, or reactions to physical and physiological concomitants of the disorder.

Fourth, experimenter bias is an ever-present source of difficulty in behavioral studies and should be minimized by appropriate experimental procedures at all levels of assessment.

Fifth, the criteria and procedures used in determining the chromatin or karyotypic status of individuals also need greater attention. It has been pointed out that the number of cells counted to determine karyotypes vary considerably among laboratories, making it difficult to establish indices of intra- and interlaboratory reliability (70). Also, the fact that a low base rate occurs for many chromosomal disorders suggests that small errors in karyotyping procedures may have important effects on published incidence figures (100). The problem of mosaicism may be important in this regard. Mosaicism cannot be ruled out without the karyotypic examination of different tissues. Reliance on sex chromatin examination alone may underestimate the extent of mosaicism (12). There are a number of technical factors that may influence sex chromatin counts, e.g. source of specimen, preparation and staining techniques, number of cells counted, etc

(98). The possibility that sex chromatin counts may be related to length of institutionalization (104) needs to be explored further since it implies that drug treatments and other alterations of metabolic activity may produce variations in the results of sex chromatin surveys.

Many conceptual issues need clarification; three will be discussed briefly here. First, it is almost invariably assumed that a deviant chromosomal constitution alters psychological development abnormally. Virtually no one has looked for positive adaptive consequences, although it has been pointed out that if some XYY males are taller and bigger than their peers, they may, on the bases of compatibility or similarity, be more likely to be attracted to, and model or imitate other taller males (70). There is evidence that taller males are more popular than average and have a higher probability of becoming leaders with high self-esteem during their adolescent years. Shah (100) has suggested that there may be an increased frequency of males with XYY complements among tall and socially assertive persons who have attained economic success and have made outstanding social adjustments and contributions. Non-DS translocation carriers of the D/G or G/G type may possess a biological advantage in that they may outnumber family members who are not carriers (4). Finally, Money & Mittenthal (17) have indicated that TS patients may "adapt to life much better than might be expected considering the problems they face," and conjecture that the genetic defect that produces TS may also produce a beneficial effect on personality. The persistent focus on abnormalities may seriously compromise the objectivity of investigators.

Second, the accuracy of the notion that individuals have fixed personality traits that determine their behavior in a variety of different settings is increasingly being called into question. Recent studies show that consistent differences among situations and situations by person interactions account for as much or more of the variance in different types of behavior as consistent differences among people. This body of literature suggests, at least for personality and behavioral data—if not also for intellectual data, that the setting in which an individual is assessed may contribute importantly to the specific results and conclusions (105–107). There is, for example, extensive evidence that various indices of aggressive behavior vary substantially from one environment to another (108).

Third, almost all investigators agree that an extreme degree of phenotypic variability exists among individuals with the "same" chromosomal anomaly. This variation must, in part, be related to differences in the environment in which these individuals have been raised and in which they presently function (i.e. gene-environment interactions). Few, if any, systematic assessments of either intrafamilial or institutional environments have been carried out. The rudimentary description of environment as either harmonious or inharmonious is simply too inadequate to be useful.

A large literature on different methods of conceptualizing environments now exists which just cannot continue to be ignored in human behavior genetics research. This issue cannot be discussed in detail here; however, Moos (108) has recently presented six major methods by which human environments can be conceptualized and assessed. Environments cannot be ordered along a single favorable-unfavorable continuum, since, for example, an environment favorable for the development of independence and self-reliance may differ from one that is favorable for the development of social conformity or abstract thinking (109). Environment assessment is relevant for both the institutional milieu, e.g. hospital wards and institutional units, and for the "developmental" milieu in which the individual is raised. For example, different psychiatric wards, even within the same hospital, differentially reinforce the open expression of anger and aggression (110). Thus, for example, XYY individuals may differ in behavioral expression of anger and aggression in institutions in part because the social climate in their units differentially rewards the expression of anger. Perhaps most pertinent for behavior genetics research are methods of measuring social climates and functional or reinforcement analyses of environments. In the latter method the specific stimuli or stimulus complexes which reinforce or inhibit specific behaviors are carefully analyzed. Environmental conditions that are likely to influence the development of general intelligence and/or academic achievement include the climate created for achievement motivation, the opportunities for verbal development, the nature and amount of assistance provided in overcoming academic difficulties, the level of intellectuality in the environment, and the kinds of work habits expected of the individual (111). Techniques for assessing these variables are available, and environments for the development and maintenance of such characteristics as dependency, aggression, and dogmatism could be similarly delineated and measured.

Issues raised by the evidence on the effects of different environments on human adaptation include the question of the malleability of some of the specific characteristics associated with chromosomal anomalies. The behavioral abnormalities associated with chromosomal disorders are often conceptualized as immutable. Thus, in relation to XYY males, it has been suggested that repeated and prolonged attempts at rehabilitation and treatment have not been successful (69). Since powerful behavior change techniques have only recently been developed, rehabilitative failure may suggest necessary changes in rehabilitative techniques rather than predictions of future failure.

In this connection, the effects of a matrix-language training program with DS subjects have been explored (112). An experimental group which received this training made significant gains in the language and concept areas of sentences, total language, differences, similarities, missing parts, and total concepts, suggesting that this procedure for the training of language and concept formation in these individuals was feasible. A number of other behaviorally oriented procedures have been utilized to change various specific behaviors of mentally retarded subjects (113), and have more general utility.

Swanson & Stipes (114) discuss androgen-replacement therapy in the adolescent or young adult with DS. This treatment facilitates the development of secondary sexual characteristics and decreases gynecomastia, thus allowing for the development of more normal interpersonal relationships and assertive and goal-directed behavior. The androgens may also increase libido, aggressiveness, and social drive but appear to be ineffective in older adults. Aggressive and erratic behavior may be side effects of such treatment (114). Finally, Caldwell & Smith (64) maintain that several clinical features may assist in the detection of patients with KS during the childhood period, including school and/or behavioral problems, a slim build with relatively long lower extremities, and somewhat small external genitalia. They recommend age-appropriate physiologic testosterone replacement therapy in order to provide a more normal adolescent growth and development and to prevent most of the characteristics of the older patient with KS. Thus, there is evidence that specific forms of treatment, both behavioral and physiological, may ameliorate certain symptoms associated with chromosomal abnormalities. Efforts to help chromosomally abnormal individuals should receive more emphasis and study.

## CONCLUSIONS

With the possible exception of the space-form deficits found among some TS subjects, no typical behavioral characteristics are associated with a specific chromosomal disorder. Intellectual and personality measures show a broad range of variability in the major karyotypic disorders. The sex chromosome abnormalities are not incompatible with normal intelligence; nevertheless, in each there may be a higher than average risk for retardation. Sampling issues render the available evidence equivocal on this point. Males with an extra Y chromosome do not appear to be hyperaggressive, and the evidence suggesting the presence of an increased risk for criminality seems to be no longer as strong as it first appeared. Future research needs to consider the problem of gene-environment interactions in the production and maintenance of behavioral dysfunction. Prospective study of individuals detected with chromosomal disorders at birth are in order, and the assessment of institutional and familial environments is strongly needed. The possibility of adaptive behavioral consequences associated with chromosomal disorders should also be explored.

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# ENTEROTOXIC[1] DIARRHEAL SYNDROMES

*7064*

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Diarrheal disease of bacterial origin is a worldwide problem and no population is spared. In geographical areas where malnutrition and crowded living conditions exist, enteric diseases represent not only a striking cause of morbidity but also of mortality. However, even in places where the disease is highly endemic, recognized pathogens are isolated from less than 50% of sick individuals (1).

At present, two general mechanisms are recognized by which enteric pathogens cause diarrhea. In the first, as exemplified by bacillary dysentery, the pathogen invades the epithelial cells of the intestinal mucosa as a necessary step in the disease process (2). In the second, of which cholera may be considered the prototype, the organism multiplies in the lumen or on the surface of the epithelium of the small intestine, and exerts its effect by producing a toxin which causes the small intestinal epithelial cell to secrete electrolytes and water. The purpose of this discussion is to summarize recent advances in understanding disease caused by such enterotoxin-producing organisms.

## CHOLERA

Less than twenty years ago an accepted view of the pathogenesis of asiatic cholera involved a desquamation of the small intestinal epithelium of the host (3) by a mucinolytic enzyme elaborated by *Vibrio cholerae* (4). The voluminous amounts of fluid observed in the classical form of this disease were considered to pass into the intestine via this denuded mucosa. By the early 1960s this concept was rejected on the basis of the observations of a number of investigators. These observations included: (*a*) The intestinal epithelium remains intact in both human (5) and experimental cholera (6); invasion of the mucosa is not a feature of the process (7). (*b*) The cholera vibrio elaborates an enterotoxin which by itself is capable of eliciting fluid loss into the small intestine of experimental animals (8–10) and of man (11). (*c*) The protein concentration of the diarrhea stool is much lower than one might expect had the fluid come from a denuded intestine. This fluid is isotonic and contains approximately twice the concentration of bicarbonate and four times the concentration of potassium of normal plasma (12).

Spurred by the current pandemic in which cholera has spread from the Far East to the Middle East, Russia, and other parts of Europe and Africa, rapid and substantial progress has been made in both basic and practical aspects of this problem. The toxin has been purified (13, 14), crystallized (15), and determined to be a heat- and acid-labile protein with a molecular weight of 84,000 (16) which is destroyed by pronase but not by trypsin. All available evidence suggests that there is only one antigenic type of cholera enterotoxin (17), even though three established serotypes of *V. cholerae* are recognized.

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[1] Due to space limitations, staphylococcal enterotoxin is not covered in this review.

With the availability of purified enterotoxin, a wide variety of investigations have been designed to elucidate the mechanism of fluid loss in the disease. Suggestions that cholera-induced diarrhea results either from an increase in the permeability of the small intestinal mucosa or from an elevated hydrostatic pressure in the lamina propria have been considered, but neither has been supported by laboratory studies (18–20). Similarly, the concept that fluid is lost solely because of impaired absorption of sodium from the lumen (21, 22) has not been substantiated. On the contrary, stimulated by this latter work, investigators have accumulated data which indicate that fluid loss is associated with an active secretory process. While studies of unidirectional and bidirectional sodium fluxes carried out in man (23, 24) and in intact animals (25, 26) have yielded some contradictory results, in vitro studies employing isolated viable strips of mucosa mounted in Ussing chambers have been most productive. These studies indicate that cholera toxin acts on the epithelium to cause a decreased active absorption of sodium and an increased active secretion of chloride (27–29). If such a process occured in the intact host, it would result in net water and electrolyte loss into the bowel lumen as is observed in cholera.

These experiments employing the Ussing chambers have indicated that the response of the mucosa to cholera toxin with respect to fluid and electrolyte transport is similar to that previously observed following exposure of the small intestine to cyclic 3′,5′-adenosine monophosphate (cAMP) (30). One difference which has been noted is that the response to cAMP is rapid while that to cholera is delayed. Nevertheless, this important observation has led to the concept, strongly supported by various experimental approaches, that the physiologic disturbances in cholera are mediated through cAMP, or by a mechanism whose end result is similar to that of enhanced cAMP activity. Direct evidence to support this thesis thus far demonstrates that increased levels of either cAMP or adenyl cyclase (which is involved in the synthesis of cAMP) are present in the small intestinal epithelial cells of animals exposed to cholera toxin (31–33). In addition, Chen et al (34, 35) have found that mucosal biopsies obtained from patients with clinical cholera contain approximately twice as much adenyl cyclase as similar material taken during convalescence.

The present working hypothesis derived from these studies is that cholera enterotoxin stimulates the production of adenyl cyclase which in turn produces elevated levels of cAMP in the intestinal epithelial cell. This results in a loss of fluid into the lumen of the bowel. Efforts are now being made to find substances which will interrupt this chain of events. One such compound, ethacrynic acid, thought to inhibit adenyl cyclase (36), has been effective in reducing fluid secretion from the intestine of dogs exposed to cholera enterotoxin (37). Cyclohexamide is another agent which has a role in inhibiting fluid secretion from the enterotoxin-exposed intestine (38–41); this compound is thought to inhibit protein synthesis in mucosal crypt epithelial cells which may be the site of production of fluid in cholera (42). However, it is not yet apparent that the synthesis of adenyl cyclase is inhibited by cyclohexamide, and thus its precise mechanism of action is not known. Should more effective compounds be found which would be suitable for use in man, a new approach to the treatment of cholera and diarrheal diseases of other etiologies (see below) will be at hand.

Until new therapeutic regimens for cholera are available, the traditional but effective use of parenteral fluid replacement remains the standard treatment for this disease. Two developments, however, have allowed some modification of the classical therapeutic approach. First, the use of tetracycline, chloramphenicol, furalazine, or furazolidone significantly reduces the duration of diarrhea, the period of pathogen excretion, and the amounts of fluid required for therapy (43–45). The second modification takes advantage of the fact that glucose-stimulated sodium and water absorption are not impaired in cholera. Thus, as an adjunct to intravenous fluids, glucose-containing electrolyte solutions given orally have been effectively employed not only in treating cholera but also in other infectious diarrhea (46–50). Both of these modifications offer the advantage of sparing intravenous fluid supplies which tend to be scarce in epidemic situations.

The prevention of cholera obviously remains a long term objective, but until better public health facilities are universally available, personal hygiene and vaccination are the only tools for its control. The immunizing agents in common use consist of killed bacterial cells and do not by design contain toxin or toxoid. The results of controlled field studies employing such products confirm that they do offer a statistically significant degree of protection (51–53); however, there is much room for improvement. Recent experiments employing laboratory models have shown parenteral administration of cholera toxin or toxoid protects animals against challenge with virulent bacilli (17, 54, 55). Because of such results, much effort is now being expended to prepare a toxoid from cholera enterotoxin which is acceptable for use in human beings. One can only hope that such a product will prove to be a significantly better immunizing agent than vaccines currently in use.

### *Escherichia coli*

There is growing evidence that some strains of *E. coli* can cause acute diarrheal disease in children and adults as well as in newborns. These strains provoke diarrhea either by a dysentery-like process (invasion) or by a cholera-like mechanism (enterotoxin) (56–60). Patients stricken with the invasive type of disease commonly exhibit fever, abdominal cramps, and watery diarrhea, followed shortly thereafter in many cases with typical dysentery (bloody mucoid stools) at which time urgency and tenesmus are described. Abnormal numbers of inflammatory cells are present in the feces. Recently, over ninety separate outbreaks due to imported cheese infected with invasive *E. coli* (O:124) have been reported in the United States (61). Other *E. coli* O-groups which have been associated with the invasive type of disease are: O:28, O:112, O:115, O:136, O:143, O:144, O:147, and O:152. In distinct contrast, noninvasive, toxigenic *E. coli* groups generally produce diarrhea of varying severity, with minimal fever, cramps, or inflammatory reaction.

Knowledge concerning the biology of enterotoxin-producing *E. coli* has been obtained with strains isolated from both swine and calves (62–65) as well as from human beings. Both a nonantigenic heat-stabile and an antigenic heat-labile toxin are elaborated by many strains and the relationship of one to the other is unclear. At the present time *E. coli* enterotoxin has not been separated from cellular constituents (66). Until such purification is achieved, the precise nature of its composition and mode of action remains obscure.

In spite of this lack of success in purifying *E. coli* enterotoxin, studies are continuing in other areas. Similarities in the response of experimental animals to either cholera or *E. coli* enterotoxin have been noted by several investigators. The characteristics of fluid secretion with regard to electrolyte content, and the microscopic anatomy of the intestinal mucosa, are identical following exposure to either *E. coli* or cholera enterotoxin (67). As with cholera, fluid secretion following exposure to *E. coli* toxin is associated with rises in adenyl cyclase in the mucosa (68). Furthermore, an interesting immunologic relationship between cholera toxin and *E. coli* toxin has been reported by Gyles (69, 70). He has found that high dilutions of cholera antitoxin neutralize the fluid-evoking capacity of the *E. coli* enterotoxin; however, the reciprocal was true only when very low dilutions of *E. coli* antitoxin were employed. Similar observations have been made by Sack & Smith (71). The conclusions reached from these studies are that the host response to cholera and *E. coli* enterotoxins are similar, and that an antigenic relationship exists between cholera and at least some *E. coli* toxins. The latter finding is significant and raises the possibility that should cholera toxoid prove to be effective in man, it may also offer a degree of protection against some *E. coli* toxigenic strains.

In an outstanding series of studies Smith and co-workers (72) demonstrated that the ability to elaborate enterotoxin is transmissible from some porcine strains of *E. coli* to nontoxigenic enteric bacteria by a conjugal mechanism similar to that of the antibiotic resistance transfer (RTF). More recently a comparable situation has been shown to exist with some strains of human origin (73, 74). These findings immediately raise the specter of widespread genetic transmission of toxin-producing capability, thus rendering nontoxigenic strains pathogenic. Under present conditions this should not be a cause for immediate concern for two reasons. First, there is no strong selective pressure to favor the survival of toxin-determining plasmids such as the manner by which widespread use of antibiotics have perpetuated R-factors. Second, the mere ability to produce enterotoxin does not in itself render *E. coli* pathogenic. The organism must, in addition, be able to multiply in the small intestine (59). In the case of porcine strains, possession of the K-88 antigen has been demonstrated to be of major importance in conferring pathogenicity (75), perhaps by allowing the bacteria to adhere to the intestinal mucosa (76). A comparable relationship has not yet been recognized in strains of human origin. Some of the latter belonging to the following *E. coli* O-groups have been shown to elaborate enterotoxin: O:06, O:15, O:26, O:55, O:78, O:111, O:119, O:125, O:128, and O:148. It should be stressed, however, that serotyping is at present of no value in identifying enterotoxigenic strains. Biologic tests in laboratory animals are the only procedures currently available for detecting toxin production.

Although toxigenic *E. coli* have been shown to be of significant importance in veterinary medicine, their role in the overall picture of diarrheal disease in man is only now being defined. Much of our present problem resides in the difficulty of detecting toxigenic strains, since animal tests which are neither easily performed nor easily interpreted are required for diagnosis. However, studies carried out on children in Chicago (77) and among Harvard University personnel traveling to underdeveloped countries (78) have given support to the idea that enterotoxin-producing *E. coli* strains may be responsible for a significant proportion of previously undiagnosed diarrheal disease.

### *Clostridium perfringens*

*Clostridium perfringens* is one of the most prominent agents involved in food infection in the United States (79). The disease produced by *C. perfringens* is usually a mild diarrhea of short duration. Recently a heat-labile enterotoxin has been described in some strains (80, 81), and the ability to produce disease in experimental animals (82) and in volunteers (83) has been correlated with the property of enterotoxicity. In the latter study it was demonstrated that cell-free material also could induce signs of disease in some individuals, but true *C. perfringens* food poisoning (as opposed to food infection) is not considered to be a frequent occurrence. Products from some strains of the organism have been shown to stimulate adenyl cyclase activity in frog erythrocyte membrane (84), but there is no further evidence indicating that *C. perfringens* enterotoxin acts on the small intestine in a manner similar to that of *V. cholerae* and toxigenic *E. coli*. However, one must consider this to be a distinct possibility.

With the realization that enterotoxins are important in the pathogenesis of diarrheal diseases caused by *V. cholerae*, *E. coli*, and perhaps *C. perfringens*, other species suspected of producing this syndrome have been examined for similarly acting toxins. Thus, cell-free material from *Pseudomonas aeruginosa* (85), nonagglutinating vibrios (86), and *Shigella dysenteriae* 1 (87) have been found to be active in animal models. In the case of *S. dysenteriae* 1, the enterotoxin probably acts in a manner different from that of *V. cholerae* or *E. coli* in that invasion of the mucosa is a requisite of disease production. Mutants which are toxigenic but lack the capacity to invade fail to evoke signs of disease when fed to monkeys (88) or volunteers (89).

From these studies it is apparent that many organisms previously not recog-

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# MECHANICS OF AIR FLOW IN AIRWAY OBSTRUCTION[1]

7065

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## Introduction

Airway obstruction is a cardinal symptom of lung disease. It increases airflow resistance throughout the airways, leads to increased work of breathing, and decreases maximum expiratory flow rates. This limits exercise performance and contributes, via ill-understood neural mechanisms (1), to shortness of breath. More indirectly, airway obstruction affects gas exchange. It usually affects peripheral airways and is never uniformly distributed among them. Thus, airway obstruction is often accompanied by nonuniform distribution of inspired air to the alveoli in different peripheral lung units. This in turn leads to arterial hypoxemia through mismatching of air and blood supply rates. Later, the obstruction may become so severe that total alveolar ventilation is diminished, resulting in carbon dioxide retention and respiratory acidosis.

Airway obstruction can develop from a variety of known and unknown causes including cystic fibrosis, bronchitis, bronchial asthma, byssinosis, and emphysema. Substances as diverse as cigarette smoke (2), textile dusts, and toluene diisocyanate vapor (3) can induce acute reversible airway obstruction and it is very likely that repetition of acute insults over the years can lead to chronic irreversible obstruction. Therefore, the finding of airway obstruction should lead to a diligent search for etiological factors rather than to an umbrella-type diagnosis like chronic obstructive lung disease.

Even though interest in airway obstruction has markedly increased during the past few years, we still lack insight into the natural history of the different diseases associated with it. Observations in symptomatic patients usually concern late or endstage disease, where etiological factors have been forgotten or are difficult to ascertain. Although early functional defects indicating airway ob-

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struction can be demonstrated in teen-age and college-age smokers (4, 5), the further progression of this defect has not been studied. Therefore, we do not know to what extent function continues to deteriorate as young people continue to smoke. It probably does continue to deteriorate, since severe airway obstruction is common among older persons with a lifelong history of heavy smoking, but there are no studies demonstrating whether and how the early stages develop into the later ones. Airway obstruction may persist for long periods of time after acute viral infections (6) but the lack of baseline pre-infection data makes the evaluation of this finding difficult. More information on the functional sequels of these common infections is needed.

Chronic airway obstruction is not always irreversible. Cessation of smoking (7) and administration of bronchodilator drugs (8) can improve function even in older persons. Avoidance of occupational dust exposure may at least halt further functional deterioration. Atropine and other anticholinergic drugs may improve function in patients who do not respond to sympathicomimetic drugs (9).

Our topic has been the subject for many semantic battles. There is considerable disagreement on the definition of chronic bronchitis, emphysema, and asthma. We shall not join this semantic battle since we agree with Scadding (10) that the concept of a disease is merely "a convenient logical device by which defined groups of patients can be referred to clearly." For our purposes we propose the following simplified definitions of common forms of obstructive lung disease. We believe that these can be used as "baseline" definitions, to which appropriate qualifying statements may be added for specific purposes.

Asthma = reversible airway obstruction, with smooth muscle constriction and secretion of tenacious mucus;

Bronchitis = hypersecretion of airway mucus and the consequences thereof;

Emphysema = disruption of lung parenchyma and gas exchange surface area, and the consequences thereof.

The pathologist can, for instance, qualify the definition of emphysema by adding specific anatomical lesions that can be measured in tissue specimens. The physiologist uses a physiological definition of parenchymal destruction; this includes loss of lung elastic recoil, indirectly measured from the pressure in a balloon in the esophagus. These definitions are useful tools in the description of phenomena that can be observed at the bedside, in the laboratory, or in the field survey.

Our review has been written to provide the physician with a framework of physiological knowledge about airway obstruction. Hence, full coverage of the literature has not been attempted. Many topics which we only touch upon have been discussed in detail in recent symposium volumes (10–13).

## NORMAL AIRWAY FUNCTION

Airway obstruction implies an impediment to the flow of air in and out of the lungs. To describe these impediments we first discuss air flow under normal conditions. These differ considerably in the various parts of the conducting airways, which we shall divide into extrathoracic (nose, mouth, pharynx, larynx, and extrathoracic trachea) and intrathoracic (extrapulmonary and intrapulmonary). The intrathoracic trachea and the main stem bronchi are extrapulmonary; all other bronchi and bronchioles are intrapulmonary. Detailed anatomical descriptions of the bronchial tree are available (14, 15).

*Extrathoracic airways.*—The flow of air in the extrathoracic airways is determined by 1. the pressure drop between the atmosphere and a point in the airway where it enters the chest, and 2. the caliber, shape, and length of the airway. On inspiration, the pressure within the airways is less than atmospheric. Physiologists assign zero value to atmospheric pressure; a subatmospheric pressure is therefore called a "negative" pressure. Negative pressure is generated by the inspiratory muscles, largely in response to the ventilatory demands, and this force is increased during obstruction.

Most anatomical features of extrathoracic airways are fixed, but two principal mechanisms, dynamic compression and glottal aperture, are variable. Dynamic compression is easily understood if one observes the nose during forceful inspirations. With rapid inspiration, due to the resistance within the external nose, the pressure inside the nares becomes sufficiently negative to overcome the elastic recoil of the alae nasi and the cartilaginous portions of the external nose cave inward. They are compressed by a transmural pressure difference, and the compression is called "dynamic" because it happens only while air flows. When inspiratory effort is further increased, inspiratory flow may become constant, limited by the dynamic compression of the nose (16). By itself, increased inspiratory force always results in increased flow. By itself, dynamic compression always results in decreased flow since it narrows the caliber of the nares. Together, increased inspiratory force and dynamic compression result in a constant flow rate independent of inspiratory force once a maximum flow has been reached.

Dynamic compression on inspiration can also occur in the extrathoracic trachea, especially when the tracheal wall is weakened by disease. The cricoid cartilage, a complete ring of cartilage around its upper end, tethers the trachea and helps keep it open. Although the membranous portion of the tracheal wall can easily invaginate when a compressing transmural pressure develops, forceful inspirations are normally not powerful enough to cause substantial dynamic compression. But when there is obstruction upstream from the trachea (e.g. in the nose), large inspiratory pressure drops develop across the extrathoracic airways, and the intratracheal pressure may then drop sufficiently to cause dynamic compression. By a quite similar mechanism in asthma, bronchitis, or emphysema, the development of dynamic compression is promoted in large intrathoracic airways during expiration (see below). The main difference is that dynamic compression of extrathoracic airways occurs during inspiration while compression of intrathoracic airways occurs during expiration.

The aperture of the glottis varies with the phase of breathing and with phonation. During speech, the glottis is wide open during inspiration and during production of consonants; it is periodically closed during production of vowels. Precise measurements of glottal aperture are difficult since the vertical position of the larynx, the degree of tilt of the vocal folds, and the shape of the glottal chink are all subject to change. The surface area of the glottal opening can be measured from high speed movies during various speech sounds (Lieberman 17). With a simpler technique, Stanescu et al (18) found that the aperture of the glottis decreases during expiration from 80% of total lung capacity (TLC) to residual volume. Since the potential changes in glottal aperture are large, large changes in total airway resistance can result.

Airways between the nose and subglottal area are irregular in shape and dimension. Air flow through them is not altered in direct proportion to pressure drop and is best described in terms of nondimensional constants, the Reynolds' number, and the coefficient of discharge (19). The resistance of these airways varies with flow and can be calculated if subglottal pressure is measured through a catheter passed through the glottis, or through transtracheal puncture (20–22). Nasal flow resistance, used for clinical assesssment of nasal obstruction under a variety of circumstances, is estimated using an oral catheter for pressure measurement. The upper airways, including the glottis, account for over 50% of the total resistance to air flow if breathing is through the nose, and for over 40% if breathing is through the mouth (20).

*Intrathoracic airways.*—During inspiration, pressures in the airways and the chest cage are negative relative to atmospheric pressure. Hence, inspiratory forces exert a potential widening force upon the airways, over and above the widening which results from increased elastic recoil as lung volume increases during inspiration. During expiration, airway caliber decreases as lung volume decreases, but in addition dynamic compression plays a large role in further decreasing the caliber of large intrathoracic airways, especially during forced expirations.

*Inspiration:* Several investigators have studied air flow patterns in the trachea and lower airways, using direct pressure measurements in man (21) or experiments with physical or mathematical models (23–26). The inspiratory flow pattern depends to a large extent on entrance conditions such as the glottal opening (26). The branching angles of the bronchi are also important determinants of flow patterns and resistance (27). Additional complexities are introduced when the walls of the airways are covered with abnormal amounts of mucus; at high rates of airflow, the layer of mucus may break up and create additional resistance to flow (28). However, by far the most important factor determining distribution of resistance along the bronchial tree is the great increase in total cross-sectional area from the trachea to the alveoli via the successive generations of branching bronchi. Early investigators, including Einthoven (29), believed that most resistance to flow was in small airways such as bronchioles because of their minimal diameters. However, bronchioles are not only minute in size, but also, since there are so many of them, airflow through each is also very small. The aggregate cross-sectional area of 16th generation bronchioles is of the order of 175 $cm^2$ and that of respiratory bronchioles increases to about 370 cm$^2$, while the cross-sectional area of the trachea is only about 2.5 cm$^2$ (15). When aerodynamic theory is applied to a model of the airways that conforms to this anatomical configuration, the predicted total inspiratory resistance is close to values measured in healthy man (24). Macklem & Mead (30) partitioned the pressure drop along the bronchial tree and found, in isolated lungs, that airways smaller than 2 mm in internal diameter contributed very little to total resistance. Model studies have led to similar results (25) and it is now generally accepted that in the healthy human bronchial tree, the trachea and first few generations of bronchi are the major site of airway resistance. This has important consequences for the interpretation of measurements of airway resistance (see below).

*Expiration:* During quiet breathing expiration is largely passive, with the alveolar driving pressure provided by the combined elastic recoil of the lungs and chest wall, the flow modulated by persistent activity of inspiratory muscles (31), and resistance offered by the glottis opening (17, 18). Pleural pressure remains subatmospheric. To increase expiratory flow rate, alveolar pressure (the sum of pleural and elastic recoil pressures) is increased by contraction of expiratory muscles supplying the additional force. The pressure rises throughout the chest cavity, in the pleural space, in the space surrounding the intrathoracic airways, and in the alveoli. At points downstream from the alveoli, in the direction of the mouth, pressure decreases because energy is dissipated by flow. During forced expirations, because of the fall in intraluminal airway pressure, the pressure inside large intrathoracic airways becomes less than the pressure outside their walls, and these airways are subject to a compressing force. Their compressible walls become narrowed, a process called dynamic compression. The mechanical relations are complex; the exact pressure differences across the airway walls cannot be measured, and the degree of compressibility of various portions of the bronchial tree is not known in detail. The physiological principles involved have been reviewed elsewhere (32) and only an outline of relevant publications is discussed here.

That large airways become compressed during forced expirations was first recognized by Einthoven (29). Dayman (33) recognized that dynamic compression would limit expiratory flow rates, and the work of Fry et al (34, 35) provided an important baseline for studies of the intricate pressure-flow-volume relations which result from the curious fact that the expiratory bellows is located within the expiratory pump. More recently Pride et al (36) have emphasized the compressed airway segment, while Mead et al (37) have focused on the events in the peripheral uncompressed airways. The following synthesis is a simplification derived from the works of Fry (34, 35), Pride (36), and Mead (37), and presents the essence of the factors which determine expiratory flow limitation. During forced expiration the pleural pressure is positive, and the driving alveolar pressure is the sum of referred pleural pressure and pressure generated from elastic recoil of the surrounding lung. Downstream from the alveoli, intraluminal airway pressure falls as a result of loss of energy due to friction generated by flow. When air leaves the mouth it is at atmospheric pressure. At some point within the branched intrathoracic airways the intraluminal pressure is equal to the pleural pressure [equal pressure points (37)]. The fall in pressure from alveoli to these points equals the elastic recoil pressure of the portion of the lung upstream from them (since alveolar pressure equals elastic recoil pressure plus pleural pressure). The locations of the equal pressure points vary with lung elastic recoil and flow. Downstream from them, intraluminal pressure continues to fall until the transmural pressure of the airway exceeds the elastic recoil of the airway wall, at which point the airway is dynamically compressed. In normal persons, airway compression during forced expiration first occurs in the trachea near the thoracic outlet, and the compressed segment rapidly elongates toward lobar bronchi as expiratory force increases (38). Once airway compression occurs, flow through the peripheral as well as central compressed airways reaches a maximum value which is dependent upon lung elastic recoil and hence lung volume. Because the compressed segment acts as a nozzle, airflow conditions within it are highly unstable and the walls tend to flutter (34, 35); hence the noise generated on forced expiration. Because small and large airways are in series, the total flow through the aggregate small and large airways must be equal. Therefore, during dynamic compression flow is maximal in small as well as in large airways. The resistance of airways upstream from the equal pressure points equals the elastic recoil of the lung specific for a given lung volume divided by the maximum expiratory flow specific to that lung volume (37). At resting lung volumes, upstream resistance approximates 2.0 cm $H_2O$/liter/sec; this seemingly high value reflects that it is measured during maximum flow. The importance of upstream resistance lies not so much in its numerical value, but rather in the concept that the stage for expiratory flow limitation is set by the lung elastic recoil and the upstream airway resistance. The more dramatic event of airway compression in the large airways is to a large extent a consequence of the conditions in these upstream airways.

In healthy subjects, maximum expiratory flow rates become limited by dynamic compression at lung volumes approximating 70% of the vital capacity (37). Below this volume, maximum expiratory flow becomes independent of effort in that once maximum flow is reached, further increase in effort will not result in systematically higher flows. Measurements of these maximum flow rates reflect important mechanical characteristics of airways during expiration, and because they are effort-independent, alveolar pressure measurements can be dispensed with for many clinical purposes.

The two main factors determining maximum expiratory flow are 1. the caliber, and thus the resistance to flow, of small airways; and 2. the elastic recoil pressure of the lungs (37). In addition, the compressibility of large airways plays a role which is probably minor in healthy individuals (36, 39–42). However, the relative importance of each of these three factors causing decreased maximum flow rates in disease is difficult to determine since only indirect methods can be used in vivo (see section on lung function tests). In principle, measurement of lung elastic recoil and maximum flow rates provide information on the resistance of the small airways which comprise the "upstream airway segment." Measurements of total airway resistance, on the other hand, mainly reflect the caliber of the large airways and the resistance of upper airways (25, 30). Together, measurements of airway resistance, maximum expiratory flow rates, and lung elastic recoil provide information which allows an indirect assessment of airway obstruction in terms of involvement of large and small airways. The terms large and small airways in this context are anatomically not precisely defined. Large airways include at least the trachea, and main and lobar bronchi, while small airways may include respiratory bronchioles and bronchioles, and perhaps also the last generation of small bronchi.

*Distribution of ventilation:* Inspired air is transported through the conducting airways by bulk flow (mass movements) and diffusion; the latter becomes increasingly important in small airways, bronchioles, and alveolar ducts. Experiments with heavy and light gases have clearly shown the importance of diffusion as a transport mode (43, 44). Some authors believe that diffusion limits the extent to which inspired and alveolar gas are mixed within the primary lobule (alveolar duct+alveoli) in normal lungs (45). We agree with Rauwerda (46) and Staub (47) that the available data are compatible with diffusion being rapid enough to mix inspired and alveolar gas nearly completely within the normal duration of inspiration. However, in diseased lungs, diffusion limitation may be an important mechanism limiting gas exchange (48).

The regional distribution of inspired gas to the lungs depends on two main mechanisms: 1. gravity-imposed variations in lung expansion, and 2. time-constant distribution. The effect of gravity can be appreciated if one considers Milic-Emili's model of a spring, held vertical at its upper end. The upper coils are stressed and pulled apart by the weight of the spring while the lower coils at first remain close together. A similar expansion pattern occurs in the normal vertical lung when expanded from residual volume. First, the alveoli in the upper lobes are expanded more than those of the lower lobes. At resting end-expiratory lung volume, or functional residual capacity (FRC), the alveoli in the lung tops are much larger than those in the lower zones (49). Above FRC this reverses and the alveoli in the lower lobes begin to expand more (50). Thus, when inspiring from FRC, the lower lung zones normally receive more of the inspirate than do the lung tops. The second mechanism, time-constant distribution, is the product of resistance and compliance, and has the dimension of time:

$$\{ R \times C = (\text{cm} \times \text{sec/liter}) \times (\text{liter/cm}) = \text{sec} \}$$

The higher the resistance or the more compliant the unit, or both, the longer the time required for air entry. In normal lungs, while time constants may not be equal everywhere, they are small and therefore unimportant in determining distribution (30). But in lungs with obstructions in small airways, an uneven increase in time constants is probably a major factor in determining gas distribution. Because of the large number of airways and their differing diameters and lengths, it is highly unlikely that obstruction would be distributed uniformly. As a result, in the presence of airway obstruction large differences in time constants would be expected between airways even within the same lung segment. The resultant maldistribution of ventilation is dependent upon breathing frequency. If the breathing rate were slow enough, even lung units with long time constants would have time to fill and empty; such units would fill less when breathing rate is rapid. The maldistribution of ventilation resulting from airway obstruction may be reduced by collateral ventilation at the pre-alveolar level (51). Also, lung units surrounding a partially obstructed area may help increase ventilation of that area by exerting increased elastic tension on its walls. This effect, described by Mead et al (52) and later confirmed by Menkes et al (53, 54), acts to enhance the inherent stability of lung spaces within a lobar boundary.

Changes in gas distribution can be assessed with topographical (e.g. radioactive xenon) or nontopographical (e.g. nitrogen washout) methods. In the former, after rebreathing xenon (an inert radioactive gas) to achieve uniform distribution, the rate at which it is washed out from the lungs while breathing air can be determined for relatively large areas of lung by monitoring change in radioactivity using collimated scintillation detectors strategically placed around the chest. This technique gives a gross topographical picture of distribution of ventilation. The wash-in of xenon after air breathing can also be measured.

Another technique for determining distribution of inspired gas at different lung volumes uses injection of a small bolus of xenon into the inspired air stream (50). When injected near residual volume most of the xenon goes to the upper lobes of the vertical lung; above resting end-expiratory lung volume, most of it goes to lower lung zones.

In measuring the rate of nitrogen washout, if all lung units were ventilated equally relative to their initial volume, the rate at which nitrogen is washed out during oxygen breathing would be the same for all units, and alveolar nitrogen concentration would fall exponentially. Thus, a graph of alveolar nitrogen concentration (on a logarithmic scale) versus the number of breaths (or total expired volume) on a linear scale would yield a straight line. Alveolar $N_2$ concentrations cannot be measured directly but can be estimated from end-tidal or mixed expired $N_2$ concentrations. In the presence of nonuniform ventilation-volume ratios the nitrogen concentration decay is not exponential, and the degree of deviation from linearity on a semilogarithmic $N_2$ washout graph indicates the degree of the nonuniformity in ventilation. This test is abnormal when the distribution of ventilation is nonuniform even within small areas of the lungs. Changes in gas distribution have an indirect effect on dynamic lung compliance (the ratio: tidal volume/pleural pressure change, during a tidal breath) which is normally independent of breathing rate. When time constants are small enough, or nearly equal, all lung regions have sufficient time to fill during a tidal breath and flow stops everywhere at the end of inspiration and at the end of expiration. Thus, pleural pressure change during a tidal breath is equal to the pleural pressure difference measured with breathholding at the inspiratory and expiratory lung volume, and dynamic compliance equals static compliance. When time constants are high in some parts of the lungs, air entry into those parts is delayed and the extent to which they fill becomes dependent upon the available time. Thus, if dynamic compliance is measured at increasing rates of breathing, lung regions with high time constants receive less air, and the total inspired tidal volume for a given pleural pressure change drops. Thus, dynamic compliance decreases with increasing breathing rate (55).

When small airways are obstructed, lung units normally ventilated through these airways may still be ventilated through alveolar pores or other connections between adjacent lung units. This mechanism, collateral ventilation, has recently been reviewed exhaustively by Macklem (51).

## PATHOPHYSIOLOGY OF AIRWAY OBSTRUCTION

*Extrathoracic airway obstruction.*—Nasal obstruction, compression from outside by soft tissue masses, and tracheomalacia after tracheotomy are the main causes of obstruction in extrathoracic airways. The mechanical effect of this obstruction is related to the change in caliber of the airway, change in the walls, and dynamic compression. Nasal obstruction in adults usually leads to mouth breathing so that total flow resistance does not increase. With laryngeal obstruction, or tracheomalacia, dynamic compression of the extrathoracic trachea on inspiration may severely limit inspiratory flow (56). Since the increased resistance is in an undivided portion of the airways, one expects neither nonuniformity of ventilation nor other consequences of intrathoracic airway obstruction. Accurate anatomical information (from X-ray contrast and direct observation) is essential for diagnosis and management; function data indicate neither the exact site of the obstruction nor its nature.

*Intrathoracic airway obstruction.*—The causes of obstruction of airways in the chest may be divided into mechanisms intrinsic to airways (e.g. bronchospasm, swelling of the mucosa, hypersecretion of mucus) and mechanisms intrinsic to lung parenchyma [e.g. airway obstruction resulting from reduced lung elastic recoil (emphysema)].

Smooth muscle contraction leads to narrowing of airways, but this effect may be modified by other forces depending upon the location of the airways. In small airways, the constriction force exerted by smooth muscle acts against lung elastic recoil forces which tend to keep the airways open (52–54). When these small airways narrow, the effect of constriction is amplified by folding of the mucosa (see Figure 88 in 14), while swelling of the mucosa or increased secretions further narrow the caliber of these airways. Increased mucus in airways causes increased resistance to flow at high flow rates through creation of waves by stirring the layer of viscous material, or dissociating it from the wall (28). In cartilage-containing airways the extent of narrowing of the lumen by smooth muscle contraction is modified by the cartilage in the airway walls (34, 40, 41), the cartilage acting to reduce narrowing by smooth muscle contraction. When smooth muscle tone is low, the membranaceous part of the trachea invaginates with rapid expiration, as a result of dynamic compression. However, when smooth muscle tone is high, the membranaceous part becomes much stiffer and the smooth muscle can exert enough force to pull the ends of the incomplete cartilage rings together in the trachea and cause overlapping of cartilaginous plates in lobar to subsegmental bronchi, thereby converting all these airways into pseudocomplete ringed noncollapsible tubes. Hence, while increased airway smooth muscle tone acts to narrow all airways on inspiration, during forced expirations it helps to keep large airways open. Observations on pressure-flow-volume relations in intact man support this concept. After inhalation of a smooth muscle relaxant (isoproterenol), maximum expiratory flow rates decrease in some normal persons, and this is most readily explained by increased compressibility of large airways (42). Similarly, increased airway obstruction may occur in some patients with cystic fibrosis following isoproterenol inhalation (57).

Increased resistance in small airways leads to dynamic compression of large airways during expiration at lower than normal flows. It must be emphasized again that dynamic compression of large airways is a physiological process that occurs in all healthy persons. Some clinical studies have suggested that large airway compressibility demonstrated by radiographic studies indicates "tracheomalacia" (58, 59). Since large airway compression depends on the pressure differences applied across the walls as well as on wall stiffness, conclusions concerning the latter are possible only if an airway is being compressed more than would normally be expected for the given pressure difference across the wall. Simultaneous recordings of air flow rates, intratracheal pressure, pleural pressures (from an esophageal balloon), and cineradiographic pictures of the development of airway compression during forced expirations could yield indirect information on such conditions.

The pathophysiology of emphysema varies with the type of the emphysematous lesion. In centrilobular emphysema, saccular dilatation of terminal and respiratory bronchioles is predominant, and the peripheral alveolar ducts and alveoli remain intact. Horsfield et al (60) made casts of normal and diseased lungs providing beautiful illustrations of the lesions of centrilobular emphysema. More recently, Hogg et al (61) outlined centrilobular spaces by insufflating them with fine particulate lead dust at autopsy. Since they also measured inflation pressures, they could calculate the compliance of these spaces, which they found to be reduced because at all operational lung volumes the enlarged centrilobular spaces are inflated to nearly maximal volumes. Because the compliance of these spaces is decreased (61) and their volume is large [from 0.02 to 0.44 ml at 20 cm $H_2O$ distending pressure (61)] relative to the respiratory space beyond them [0.02 to 0.04 ml (47)], frequently no inspired gas reaches the alveolar spaces beyond them. This means that ventilation of the distal alveoli is by diffusion only, thus limiting alveolar gas exchange. The consequences are arterial hypoxemia and increased dead space. In spite of the low compliance of the centrilobular spaces, the compliance of the lungs as a whole is greater than normal (61). In part, this may be due to compression of lung tissue surrounding the centrilobular spaces, so that these surrounding areas are at a lower than normal volume and therefore more compliant. The high overall compliance may also reflect panacinar as well as centrilobular emphysema.

In panacinar emphysema the major lesion is a disruption of alveolar walls, with consequent loss of gas exchange surface and elastic recoil. This leads to impaired gas exchange. The diffusing capacity for oxygen and carbon monoxide is decreased (62), which reflects the decreased gas exchange surface. Since diffusing capacity measures the effective surface area for alveolocapillary gas transfer (63), the reduction in diffusing capacity correlates well with the anatomical extent of emphysema (62, 64). Elastic recoil pressure is an important component of the driving force for expiratory flow during forced expirations, and this force is greatly diminished in emphysema, owing to destruction of the elastic support structure of the lungs (13, 65, 66). Since elastic recoil pressure is reduced, equal pressure points are close to the alveoli during forced expiration and the resistive pressure drop from them to the thoracic outlet is increased. Hence flow rate limiting dynamic compression occurs in large airways at relatively low flows. (When the elastic recoil is zero, the equal pressure points are at the alveoli, and the entire expiratory resistive pressure drop of airways within the thorax is exerted to dynamically compress the airways.) In normal lung, small airways are protected against dynamic compression by the tethering effect of lung elastic recoil (52–54), but this protection is lacking in emphysema (65). In addition, whether as an intrinsic part of the disease (66) or as a result of impaired clearance mechanisms, small airways are destroyed or are plugged with secretions (60, 65–68). Consequently, dynamic compression frequently occurs in segmental and subsegmental airways, as well as in larger airways (69–72). When this occurs the lung-emptying pattern is shifted away from the compressed airways. In small as well as large airways the relative roles of loss of elastic recoil, damage to the airway wall, and altered intraluminal pressure profiles in the course of dynamic compression cannot be determined with certainty. The mechanisms of dynamic compression probably vary a great deal depending on the extent and location of emphysema, and may vary considerably within the same lung.

The nonuniform distribution of emphysematous lesions throughout the lungs is also reflected in increased nonuniformity of inspired gas distribution. West (73) has discussed the predilection of emphysematous lesions for the lung tops, where, due to gravity, alveoli are normally under the greatest strain. Thus, development of emphysema in the lung tops may increase the normally present nonuniformity of distribution in the field of gravity (49). In addition, the increased time constants in peripheral lung units may lead to widespread nonuniformity throughout the lungs. Collateral ventilation also affects distribution patterns. If collateral ventilation is through prealveolar channels it might improve gas distribution. On the other hand, collateral ventilation through alveolar channels would have the effect of adding space ventilated in series requiring ventilation of the "trapped air space" by diffusion rather than mass air movement (51). Diffusion rates in the alveolar gas are inversely proportional to the square of the distance within the alveolar spaces. In normal lungs distances are short enough to allow virtual diffusion equilibration within the span of a normal inspiration, but in emphysema distances increase through the confluence of alveoli with destroyed walls, and this probably imposes a diffusion limit to gas transport (74).

## LUNG FUNCTION TESTS

Lung function tests are essential diagnostic and management aids in diseases characterized by airway obstruction. They do not indicate the cause of the obstruction, although hints concerning etiology can be obtained if tests are repeated under various conditions—e.g. after drugs or exposure to irritants. Some tests give information that can indicate airway obstruction; other tests also provide clues to the approximate location of the obstruction. Tests differ widely in their sensitivity to different degrees of airway obstruction and this must be taken into account when choosing the test and interpreting the results. To recognize overt symptomatic disease, relatively insensitive tests will suffice. To recognize subtle effects of environmental agents, and to follow small changes in the course of disease, more sensitive tests are required. Results must be accurate at all times; this is particularly important when the data are to be compared with follow-up data. For instance, to recognize severe airway obstruction it suffices to know that the $FEV_{1.0}$ equals 1 liter, with a vital capacity of 3 liters. To follow changes in time or with drug treatment in such a patient, differences of the order of 0.2 liter or less may be significant and should be accurately recorded. When following deterioration of function with age, as in studies on nonsmokers and smokers (75), one measures decreases of $FEV_{1.0}$ which average about 0.03 liter/year in healthy nonsmokers. Such studies obviously require painstaking care in performance of the test, maintenance of equipment, and quality control of the data.

*Forced expiratory maneuvers.*—Determination of the rate at which air can be expired during forced expirations remains the most useful test for airway obstruction. Spirometric measurements of the forced expiratory volume ($FEV_{1.0}$) and of maximum midexpiratory flow rate ($FEF_{25-75}$) are standard in most laboratories. The vital capacity, measured during the same maneuver, is referred to as forced vital capacity (FVC). Additional information can be obtained if instantaneous maximum expiratory flow rate is plotted against volume expired (the MEFV curve)—a procedure first introduced by Dayman (33) and later popularized by Hyatt, Schilder & Fry (76). This procedure has recently been used to assess airway obstruction in several clinical and experimental studies (5, 77–79).

Dynamic compression of large airways is present during maximum expiratory flow at lung volumes less than about 70% of the vital capacity, even in normal subjects (37). Although the compression of large airways determines that flow shall be limited, the rate at which it is limited depends on the supply of air from peripheral airways. Therefore, decreased maximum expiratory flow rates are associated with obstruction in small airways and are found in conditions where other tests of small airway function are also abnormal. For instance, in asthma one finds low maximum flow rates, increased nonuniform gas distribution (80), and frequency dependence of dynamic compliance (55). Potter et al (81) find that patients with severe airway obstruction reach their maximum expiratory flow rates during mild exercise. Their results suggest that comparison of expiratory flow rates relative to lung volume during tidal breathing at rest and during exercise with the MEFV curve may be useful for determining disability related to limited ventilatory capacity. The sensitivity of the MEFV curve for detecting early bronchospasm is blunted if the subject inhales to total lung capacity (TLC) prior to forced expiration, since this maneuver may by itself be enough to reverse the bronchospasm. Therefore, Bouhuys et al have used the "partial expiratory flow volume curve" (PEFV) in drug and epidemiological surveys with the subject exhaling maximally from midvital capacity rather than from TLC (77).

While MEFV curves depend upon lung elastic recoil, airway resistance, and resistance of airways to compression, they do not measure any of these parameters. Recording of alveolar pressure-flow curves at different lung volumes was first introduced by Hyatt, Schilder & Fry (76), who called these curves "iso-volume pressure-flow" (IVPF) curves. Alveolar driving pressures and lung volumes can be measured directly at low and high rates of flow and throughout nearly the full range of vital capacity with a body plethysmograph equipped with a rebreathing bag to allow the subject to breathe air preconditioned to BTPS conditions (82). The esophageal balloon method affords a less complex but more indirect method for measuring driving pressure. With it, Potter et al (81) found that patients with severe irreversible airway obstruction reached maximum expiratory flow rates at lower pleural pressures and at higher lung volumes than comparable healthy subjects, indirectly suggesting that large airway compressibility may be increased in these patients.

*Airway resistance.*—Since pressure-flow relations in airways depend on volume and are always alinear, any measurement of resistance should carefully qualify the lung volume and the rate of flow used during the test. This is done with the classical panting method of DuBois et al (83), where the airway resistance is measured at a lung volume level that is determined immediately afterwards with the plethysmograph. By performing this measurement at different lung volume levels, airway resistance can be related to lung volume. This remains the simplest and most reliable method to measure airway resistance under well-defined conditions. The panting maneuver required to decrease the effect of thermal exchange helps to keep the glottis open (18) and thus minimizes its effect on airway resistance.

Other methods for measuring resistance include use of an esophageal balloon for measuring pleural pressures to obtain pulmonary resistance (i.e. airway resistance+tissue impedance) and the interruptor (36), and the forced oscillation (84) method which provides data on total respiratory resistance, including resistance of the chest wall. Fisher et al (85) have pointed out that, if properly performed, the oscillation method is not much simpler than the plethysmographic technique. Without control of lung volume levels, the oscillation method provides less information, and recent studies have suggested that the sensitivity of the oscillation method to changes in resistance is limited (86).

Regardless of the method of measurement, airway resistance data reflect the resistance of large airways since that is where most of the resistance is normally located within the bronchial tree (30). In addition, upper airway resistance may account for as much as 40% of total resistance (20), which decreases the sensitivity of the measurement to changes caused by altered caliber of the bronchial tree. However, in conjunction with MEFV curves, airway resistance measurements can provide data on the distribution of resistance through the bronchial tree, if a crude and somewhat arbitrary division of airways into large and small is acceptable. Thus, moderately decreased maximal flow rates on the MEFV curve together with increased airway resistance would suggest narrowing of small as well as large airways. Markedly decreased maximum flows together with normal or slightly increased airway resistance suggest obstruction mainly of small airways. Isolated increases of airway resistance, with little or no change of flow rates, suggest narrowing of large airways (79).

The variability of airway resistance measurements can also be reduced when they are plotted against elastic recoil pressure (82, 87). Colebatch et al (88) have used airway conductance (the reciprocal of resistance) versus elastic recoil graphs to distinguish various forms of airway obstruction.

*Elastic recoil pressure.*—Elastic recoil pressure is the pressure prevailing in the pleural space when air flow is zero. Elastic recoil of the lungs is decreased in emphysema (64), perhaps the most direct expression of the loss of elastic structure that the lungs undergo in that disease. Measurement of lung elastic recoil requires swallowing an esophageal balloon, and the assumption that esophageal pressure sufficiently approximates pleural pressure. However, pleural pressure is not equal everywhere in the pleural cavity and is subject to a gravity gradient in upright man (49). Hence the choice of a single esophageal pressure value to reflect "the" pleural pressure at a given lung volume is beset with uncertainty. In an early study, Macklem & Becklake (89) showed that maximum static recoil pressure, i.e. lung elastic recoil at TLC, was significantly lower in patients with emphysema than in normals. More recently Jonson has shown that while this is correct, because of the large variation in normal subjects, only static recoil curves which reflect a nearly complete loss of elastic recoil can be recognized as definitely abnormal (90).

As a group, bronchial asthma patients have significantly reduced lung elastic recoil (91–93), although to a much lesser extent than do emphysema patients. The mechanism for this decrease is unknown, since acute reduction in elastic recoil has not been demonstrated in patients with asthma [e.g. during acute bronchoconstriction or after breathing at higher than normal lung volumes for periods of up to one hour (94)] and increase in elastic recoil has only been demonstrated during remission several days or weeks later (91, 92).

The principal drawback of elastic recoil measurements remains the need for an esophageal balloon. However, a newly introduced, noninvasive method designed by McKerrow and co-workers (95) may prove equally accurate. This method uses the well-known fact that skin over the suprasternal notch is drawn inward during inspiration. The extent of this displacement is a function of lung elastic recoil. If the subject exerts pressure against a closed shutter at the mouth, at a given lung volume, the displacement of the sternal notch skin is abolished, and the pressure required to do this equals the elastic recoil force.

*Distribution of ventilation*: (*a*) *topographic methods.*—Topographic methods of studying gas distribution have yielded much valuable information on the mechanics determining gas distribution in healthy subjects under various conditions of change in posture and acceleration of gravity. These methods have proven less informative for assessing the extent of abnormality introduced by disease. Perhaps this is because the methods have not been available in many laboratories, and clinical experience with them is limited. Regional ventilation distribution is abnormal in chronic bronchitics with near normal lung function (96), but not in asymptomatic asthmatics (97). The abnormal distribution as assessed by xenon did not correlate well with evidence of abnormal ventilatory capacity in these studies, perhaps indicating that the topographic distribution methods detect abnormalities not seen by other methods.

*Distribution of ventilation*: (*b*) *nontopographic methods.*—Nitrogen washout is a model of the nontopographic methods for determining distribution of ventilation. If the lung is arbitrarily divided into slowly, rapidly, and intermediately ventilated spaces, the relative size of each space can be calculated from the change in slope of the nitrogen washout curve. [No change in slope indicates all fast space; rapidly changing slope indicates large slow space contribution (98).] Change in slope of the nitrogen washout curve indicates disease in the branched airways or air spaces supplied by them. Abnormalities in the nitrogen washout curve correlate well with reduction in dynamic compliance with increasing respiratory frequency (99, 100). Measurement of fall in dynamic compliance (frequency-dependent compliance) had been advocated as a test for "small airway" disease (55). In disease states where both nitrogen washout and frequency-dependent compliance have been measured, each have been abnormal to similar degrees (99, 100), neither test holding a clear advantage over the other. It seems that since measurement of frequency-dependent compliance requires insertion of an esophageal balloon and considerable subject training in the respiratory maneuvers, nitrogen washout is the preferable method for assessing maldistribution of ventilation. As noted before, if partial obstruction of small or large airways is the cause of maldistribution of ventilation, the airway obstruction should be manifested by decreased expiratory flow rates.

Single-breath nitrogen curves (101) can also be used to assess inspired gas distribution. An increased slope of the alveolar plateau indicates nonuniform regional gas distribution as well as increased alveolar gas inhomogeneity which results from increased diffusion distances in emphysema. In recent years, the final rise of these curves at low lung volumes has received increased attention. It has been suggested that the lung volume at which this terminal rise begins (phase IV of the single-breath curve) corresponds to the volume at which small airways in dependent lung zones close (102, 103). Hence this volume has been called "airway closing volume." Interpretation of this measurement is still under discussion: there is no agreement whether closure occurs in small airways or whether it actually represents dynamic compression of larger airways (104). Airway closing volume increases with age and in cigarette smokers (102, 103, 105). Measurement of this volume has been suggested as a sensitive test for obstruction of small airways (102, 103). The results of Hyatt et al (104) suggest, however, that such measurements may yield information that corresponds to the finding of low flow rates on MEFV curves. In fact, the conditions which increase closing volume (age, smoking) also decrease flow rates on MEFV curves.

Woolcock et al (55) and Mead (106) have referred to the small airways as a silent or quiet zone in the lungs, in the sense that obstruction in these airways could be widespread and yet tests of lung function could give normal results. We believe that this concept is not solidly based. While "small airway disease" may be quiet on auscultation of the chest, Mead's own work (37) has shown that the resistance of small airways is a major determinant of maximum expiratory flow rates on MEFV curves. This concept has received strong support from several later studies which have shown that these flow rates are often significantly decreased in mild bronchial asthma and other states presumably associated with obstruction in small airways (5, 57, 77, 79). The evidence that exposure to allergens—dust, cigarette smoke, or histamine aerosol—results in small airway obstruction is indirect but persuasive, since they are accompanied by signs of increased nonuniform gas distribution, decreased steady-state diffusion capacity, and decreased arterial oxygen tension. These functional abnormalities are difficult to explain unless one assumes that the lesions are in small airways rather than in major bronchi.

## ETIOLOGY OF AIRWAY OBSTRUCTION

*Age.*—To what extent are the pathophysiological changes of emphysema a normal accompaniment of age? "Senile emphysema," a term now in disuse, used to be considered as such, without clear evidence that such an entity existed. Lung function tests show a decrement of ventilatory capacity with age, and at least a portion of this decrement can be attributed to loss of lung elastic recoil. In a population of respiratory physiologists and their children, Turner et al (107) found that elastic recoil, as measured with the esophageal balloon, decreases significantly with age. This results in lower maximum expiratory flow rates at comparable lung volumes in older subjects. Loss of lung recoil in older persons may also account for the increased airway closing volumes found by LeBlanc et al (102); diminished support of intrapulmonary airways within the elastic structure would, other conditions being equal, lead to airway "closure" at higher lung volumes. Loss of elasticity with aging may precede panacinar emphysema which has been demonstrated by Anderson et al (108) in 37% of nonsmokers dying above the age of sixty from nonrespiratory diseases. The physiologic significance of these changes remains to be seen. As man ages, he is also exposed to noxious environmental agents for longer periods of time, and it is not clear to what extent loss of lung recoil with age is an inherent accompaniment of increasing age, or reflects prolonged exposure to low level environmental agents. Since the esophageal balloon method precludes studies of large population groups, owing to its limited acceptance by subjects, only small groups have been studied. Experience with nonsmokers without occupational exposures and living in rural areas without measurable air pollution might help solve this problem, although even in such populations selection factors must be considered.

*Environmental factors.*—Epidemiologically, cigarette smoking is clearly associated with chronic airway obstruction. How this develops is less clear. Long-term studies of well-controlled populations are necessary to clearly demonstrate the relationship of any environmental factor to disease. Wilhelmsen et al (75) have performed a well-controlled study in Swedish men at age 50 and again at age 54. They show a clearly accelerated decay in timed expiratory volumes among smokers as compared to nonsmokers. However, a less well-controlled study by Howard (109) failed to show any effect of smoking. The difference is likely explained by the lack of follow-up examinations. Seely et al (5) have demonstrated reduced expiratory flow rates at 50% of the forced vital capacity among smoking high school students. Acute fall in airway conductance (2) and impaired distribution of ventilation (110, 111) have also been demonstrated following cigarette smoking.

Ozone has also been demonstrated to cause acute change in lung function. Bates et al (112) found reduction in lung conductance and maximal expiratory flow at 50% forced vital capacity following 2 hr exposure to 0.75 ppm ozone, a level which can occur in cities during periods of atmospheric pollution. These effects were aggravated by exercise, presumably because of the greater volume of air inhaled. Occupational diseases such as byssinosis and diisocyanate-induced airway obstruction are characterized by airway constriction in response to the specific dust or fume exposure (3, 79). Asthma, which may also be an occupational disease, is an important model for students of airway obstruction, since rapid changes of airway caliber occur spontaneously or may be induced with drugs. Allergy, nonantigenic histamine release, and neurogenic mechanisms are the main factors which cause airway obstruction in these diseases. Review of these mechanisms is outside the scope of this article. For recent reviews of this and related subjects see Widdicombe (113), Colebatch (114), and Szentivanyi (115).

## TREATMENT

Increased morbidity and mortality rates among patients with chronic obstructive lung disease are emphasized in the study of Jones et al (116). Any therapy which can alter the natural history of these diseases would be of great benefit.

In the following section we touch briefly on some of the therapeutic modalities in frequent use, especially where they relate to dynamics of air flow.

*Treatment of emphysema.*—If lung cysts exist as localized disease, not part of generalized pulmonary emphysema, and they are of sufficient magnitude to compress adjacent lung tissue—thereby reducing elastic recoil and hence airflow—removal of the lung cyst will result in permanent improvement in ventilatory mechanics (117). If the lung cyst exists in conjunction with or as part of generalized pulmonary emphysema, removal of the cyst may result in temporary improvement in lung function, but improvement will not be sustained for more than a few months (117). Hence, surgical removal or plication of multiple blebs in the presence of generalized emphysema is not indicated. Similarly, reconstruction of extrapulmonary airways in emphysema, to prevent their collapse, is unwarranted because the primary physiologic defect is loss of elasticity and impaired small airways function, the airway collapse being secondary (35–37, 71).

*Bronchitis.*—In a controlled study, Berrel et al (118) showed striking improvement in 2 out of 10 patients with "pulmonary emphysema" during a period of prednisone therapy. Similarly, Petty et al (119) found 18 out of 182 patients with diagnoses of emphysema and bronchitis, whose maximal voluntary ventilation increased by 30% or more while undergoing treatment in a comprehensive care program. Ten of the improved 18 patients received prednisone while, of a matching group of 21 patients who did not respond to treatment, only 3 received prednisone. Prior to treatment with prednisone, the responders could not in any way be distinguished from those who did not respond. These studies indicate that it is not possible to define "pure" emphysema or "pure" bronchitis to the exclusion of bronchospasm. For this reason we feel that a carefully monitored trial of bronchodilator and corticosteroid therapy is warranted in all patients with chronic airway obstruction who do not have purulent bronchitis. Hospitalization may be indicated during the therapeutic trial. Ventilatory function testing before, during, and after therapeutic trial is mandatory to determine the efficacy of the treatment. Astin (120) has shown fall in airway resistance while bronchitic patients with arterial desaturation breathe 30% oxygen. Campbell (121) emphasizes the therapeutic benefits of oxygen, and the hazards of withholding oxygen in the presence of severe hypoxia. Even in treatment of severe respiratory failure, life-endangering hypoventilation can usually be prevented by careful adjustment of inspired oxygen concentration and frequent blood gas determinations. The lung tissue toxic effects of oxygen concentration in excess of 50%, first pointed out by Morgan et al (122), are now well recognized.

Aerosolized water and saline solutions have been advocated in the treatment of bronchitis. Cheney & Butler (123) have shown striking increase in airway resistance during ultrasonic nebulization of water or saline in patients with asthma and bronchitis. This increase in airway resistance was not noted when the aerosol was administered using a heated reservoir nebulizer, a difference which might result from the bronchoconstrictor effect of cold droplets hitting bronchoconstrictor receptor sites (124). The role of mist therapy in treatment of obstructive lung disease remains to be established.

Purse-lip breathing is frequently advised in the treatment of obstructive lung disease. Although Thoman et al (125) have shown improvement in gas exchange during purse-lip breathing in a study of patients with chronic obstructive lung disease, their findings might have resulted from change in breathing pattern during the experimental period. Nevertheless, patients with chronic obstructive lung disease do purse their lips spontaneously when dyspneic without benefit of physician instruction. A possible explanation for this phenomenon may be that in patients with emphysema, and in some normal subjects, once flow limitation has been established at any lung volume during forced expiration, further increase in intrapleural pressure results in a paradoxic fall in expiratory flow (35, 37). This increase in intrapleural pressure can be balanced by increasing downstream resistance through purse-lip breathing, and patients may be "tuning" expiratory forces to achieve maximal expiratory flow.

Exercise has no direct effect on respiratory function (126) but does appear to improve the efficiency of peripheral utilization of oxygen and to reduce oxygen debt, and hence postexercise dyspnea. Patients who do not have congestive heart failure should therefore probably be encouraged to engage in some form of regular supervised exercise.

*Treatment of bronchospasm.*—Administration of epinephrine-like compounds and/or theophylline to patients with bronchospasm results in reduction of the bronchospasm, probably through increased concentration of cyclic AMP in smooth muscle cells (115). It also decreases frequency dependence of lung compliance, implying more uniform distribution of time constants (99). Therefore, it also should improve alveolar capillary gas exchange, but instead arterial oxygen tension falls after administration of bronchodilating drugs (127). The reason for this apparent paradox is unknown. The fall in oxygen tension may be equivalent to an arterial oxygen saturation drop of as much as 5%. Therefore, where hypoxia is of concern, it is wise to administer supplemental oxygen during and following bronchodilator therapy. In the presence of severe bronchoconstriction in which tracheal ring and bronchial cartilage overlap (39–41), nebulized isoproterenol may deposit preferentially in large airways, causing relaxation of large airway smooth muscle without altering peripheral airway obstruction. As a result, dynamic collapse of large airways could occur and the airway obstruction could appear to be aggravated, giving the appearance of "isoproterenol-induced bronchospasm." For this reason systemic rather than aerosol drug therapy may be preferable in the treatment of severe bronchospasm.

New bronchodilator agents include metaproterenol [(orciprenaline (128)], whose bronchodilator effect appears to be of greater duration than drugs available in the United States, and salbutamol, a selective bronchial $\beta$-receptor stimulant (129). Atropine and atropine derivatives have been found useful in treating refractory bronchospasm (9). Disodium chromoglycate, used extensively outside the United States, is a valuable adjunct in the treatment of asthma. This drug may act to prevent release of histamine and granules in the mast cells, and is helpful in preventing exercise-induced bronchospasm and hyperventilation-induced bronchospasm (130, 131).

Recently, phenobarbitol has been found to increase the clearance rate of dexamethasone and, therefore, to induce bronchospasm in prednisone-dependent asthmatic patients. For this reason caution should be exercised in administering barbiturates to patients with steroid-dependent airway obstruction (132). Asthmatic patients may also have bronchospasm induced by administration of propranolol, a drug which blocks $\beta$-adrenergic receptor activity. A new and as yet unreleased $\beta$-receptor blocking drug, alprenolol, apparently does not have this adverse effect (133).

## SUMMARY

A large class of lung diseases is characterized principally by airway obstruction: asthma, bronchitis, emphysema, the lung disorder in cystic fibrosis, and some occupational lung diseases. Among the etiological factors are cigarette smoking, exposure to irritant gases and dusts, allergens and age-related changes in lung function.

The physiological and clinical manifestations of airway obstruction depend upon the site of obstruction. Extrathoracic airways, nasal, laryngeal, or tracheal obstruction increase inspiratory as well as expiratory airflow resistance. During rapid inspirations, dynamic compression of the nares or of the trachea may limit inspiratory flow rates. Glottal aperture is an important physiological determinant of airflow resistance.

Obstruction of intrathoracic airways is accompanied by varying degrees of increased airway resistance, decreased maximum expiratory flow rates, increased nonuniformity of inspired gas distribution, and frequency dependence of compliance. Increased total airway resistance depends to a large extent on narrowing of large bronchi, trachea, and upper airway resistance. Decreased maximum expiratory flow rates, in particular at low lung volumes, reflect primarily obstruction in peripheral airways. Abnormal inspired gas distribution depends largely on obstruction in small airways but is also influenced by increased diffusion distances in emphysema and by collateral ventilation.

Airway obstruction in the bronchial tree results from secretion, mucosal swelling, airway smooth muscle contraction, thickening of airway walls, and loss of lung elastic recoil. The functional consequences of these pathological changes depend not only on their severity, but also on their site. For instance, airway muscle contraction narrows bronchioles but stiffens large bronchi; the former may decrease while the latter may increase expiratory flow rates.

Clinically, airway obstruction is detected by history, physical examination, and lung function tests. Measurement of maximum expiratory flow rates, using the $FEV_{1.0}$ or maximum flows on MEFV or PEFV curves, is the simplest test for quantitation of airway obstruction. PEFV curves offer a very sensitive assessment of airway obstruction with relatively simple equipment. These tests are suitable for controlled trials of drugs and other modes of therapy in obstructive lung diseases.

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# IMMUNOLOGIC RECONSTITUTION[1]

7066

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## Introduction

Immunologic reconstitution, although still in its infancy, has recently undergone tremendous growth and has been applied in many ways to the clinical management of patients with a variety of diseases. Immunodeficiency may be divided into two broad categories, primary and secondary. Primary immunodeficiency implies a failure to develop normal immune defense mechanisms. Secondary immunodeficiency, on the other hand, may result from a variety of exogenous factors (malignancy, infection, cytotoxic agents, radiation, hypercatabolism of immunoglobulins, lymphocyte depletion from intestinal lymphangiectasia, etc). The primary immunodeficiences are extremely variable and difficult to classify. For this discussion, they will be considered under deficiencies of humoral immunity, deficiencies of cellular immunity, and deficiencies of both cellular and humoral immunity (combined immunodeficiency). To date, only the latter two groups of primary immunodeficiencies have been amenable to some form of definitive correction and subsequent reconstitution of the immune deficiency. Four approaches have been used to achieve immunologic reconstitution: bone marrow transplantation, thymus transplantation, use of transfer factor, and leukocyte transfusions.

Prior to 1968, it had been possible to establish a foreign hematopoietic graft by bone marrow transplantation (BMT) in humans with aplastic anemia (1), lymphoreticular malignancy (2, 3), and marrow aplasia following irradiation (4). It was postulated in 1967 that infants with a severe deficiency of both cellular and humoral immunity represented a faulty differentiation of a lymphoid precursor cell, but possessed normal precursors for red blood cells, granulocytes, and platelets (5). Experimental evidence was accumulating which supported the concept that both pluripotential and committed stem cells reside in the bone marrow. Furthermore, transplantation of primitive stem cells could, in genetically suitable mice, prevent the mortality following lethal irradiation. It was therefore predicted that transplantation of human bone marrow from a properly selected and

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genetically suitable sibling donor might replace the deficiency of lymphoid cell precursors in infants with combined immunodeficiency disease, the so-called Swiss type agammaglobulinemia.

Initial efforts to test this postulate in infants with combined immunodeficiency disease included bone marrow and fetal liver transplantation. In several instances these procedures appeared to succeed in achieving the desired immunologic reconstitution, only to replace the certainly fatal immunodeficiency disease with an equally lethal graft-versus-host (GVH) reaction (6, 7). With the increasing knowledge of histocompatibility matching in both experimental animals and man, it became possible to visualize that with a suitably matched donor, successful marrow transplant in infants with combined immunodeficiency could be expected to correct the immunodeficiency disease while avoiding the lethal GVH reaction.

A bone marrow transplant donor is considered to be a suitable match when the donor and recipient are identical at the major histocompatibility loci. This means identical HL-A genotypes and unresponsiveness in mixed lymphocyte culture (MLC). This, with few exceptions, has been the case in HL-A identical siblings (8). The frequency of HL-A identical siblings is one in four. In rare instances when a genetic recombination occurs within the HL-A chromosomal region, HL-A nonidentical siblings can be unresponsive in MLC (9). Such was the case in our first successful BMT (10).

In 1968, a male infant with a family history of combined immunodeficiency disease in 11 male relatives over three generations was seen at the University of Minnesota. Successful immunologic reconstitution was achieved in this boy utilizing a bone marrow transplant from his sister (11, 12). The donor and recipient were HL-A nonidentical and ABO incompatible, but were well matched in mixed leukocyte culture. Today, more than four years after the bone marrow transplantation corrected his severe combined immunodeficiency disease, this child remains clinically and immunologically normal. Furthermore, it is of interest that in this first case a second BMT successfully corrected an aregenerative marrow aplasia immunologically induced by the ABO incompatibility of the donor and recipient (12). In this child who was originally blood type A, the blood type was completely switched from A to O following the second BMT, and the child to this date remains of blood group O. Thus, bone marrow transplantation has corrected both a genetically determined disease and an acquired disease based on immunologic assault. Since this marrow transplant, some fifteen additional successful transplants from matched siblings or close relatives have been performed.

Genetic markers such as those found on red blood cells and granulocytes in the marrow and peripheral blood and chromosomes on dividing cells in the marrow, as well as the complete immunologic reconstitution, clearly establish that suitably matched bone marrow transplants can correct this form of immunodeficiency and will produce long lasting reconstitution of the immunologic functions of both thymus-derived cells (T-cells) and antibody-producing cells (B-cells).

Since 1968 we have treated 12 additional infants suffering from combined immunodeficiency disease by BMT. We have achieved immunologic reconstitution in 8 of 13 patients with severe combined immunodeficiency disease. Five of these eight patients promise long term immunologic reconstitution and good health. One of the three deceased patients died when aspiration pneumonia, complicating fluid therapy for diarrhea, was fatal three months after successful transplantation and apparent full reconstitution (13). The other two children died with *Pneumocystis carinii* and cytomegalic inclusion virus disease which was almost certainly present at the time of transplant but only clinically evident when the immunologic reconstitution began. The lessons from these cases will be discussed below.

With one exception, full success of this treatment was achieved when a compatible sibling donor was available. The exception was another example of MLC nonreactivity between donor and recipient who were HL-A incompatible (14). In this patient, gradual reconstitution was achieved after three marrow transplants. In contrast, when immunologic reconstitution is attempted using incompatible marrow, a GVH reaction of varying severity, but regularly associated with fatal disease, ensues. When broader application of BMT to clinical medicine is to be realized, avoidance, modification, or control of the GVH disease must be achieved. For this reason, considerable effort has been directed toward manipulating the GVH disease encountered when major histocompatibility barriers are crossed. Excellent and exhaustive reviews are available concerning GVH disease, and in this context only a few can be mentioned with no attempt made to analyze these reactions exhaustively (15).

## IMMUNODEVIATION AND MODULATION OF GRAFT-VERSUS-HOST DISEASE

Because these patients, like immunodeficient animals, are exquisitely susceptible to GVH disease when immunocompetent cells are administered, the following techniques have been applied in an effort to minimize the GVH disease following marrow transplantation, particularly when a matched sibling donor is not available.

*Cell separation.*—It has been demonstrated that the severity of the acute GVH reactions is in a major way not only related to the differences in histocompatibility but also to the type and number of immunocompetent cells administered. Extensive work by Miller & Phillips (16) and Dicke et al (17, 18) on cell separation techniques have shown that it is possible to obtain, from a mixture of bone marrow and peripheral blood, a population of lymphoid precursor cells which are unresponsive to phytohemagglutinin (PHA) and have been called stem cells which lack immunocompetence. Using these separation procedures, it has been possible to delay the onset and reduce the severity of the GVH reaction in man. In spite of this modified GVH reaction, a high mortality persists. A further limitation of these separation procedures is that they yield few of the desired stem cells. Thus, they require a large volume of marrow to begin with, and this limits, at present, their usefulness to small infants (19). Furthermore, difficulty in establishing a bone marrow "take" in patients with combined immunodeficiency disease using stem cell preparations obtained by this in vitro separation procedure has been experienced (20). By contrast, we have observed that successful immunologic reconstitution is often possible with minimal acute GVH when a matched sibling is available or when a major histocompatibility barrier exists (21). However, in the latter case, activation and development of immunocompetent cells ultimately may lead to serious GVH disease. Thus, bone marrow fractionation achieved either by physical separation or biologically, as with the small marrow aspirates, continues to hold promise, but the threat of serious and even fatal GVH disease requires continued concern.

An alternative source of immunoincompetent stem cells for human transplantation may be found in early fetal tissues. Extensive data in the mouse clearly indicate that yolk sac cells and fetal liver cells can reconstitute both cellular and humoral immunity (22, 23). Furthermore, Bortin & Saltzstein have shown that in certain strains of mice, fetal liver and thymus cells can reconstitute lethally irradiated mice without evidence of GVH disease (24). These results are very different from those produced by using fully competent adult lymphoid cells where, in these strains of mice, GVH is the rule. Detailed studies of human fetal tissues will be important to determine if, as in the case of the mouse, cells taken early in gestation will be immunoincompetent, and thus not produce acute GVH, but still be capable of conferring immunologic reconstitution.

*Immunologic enhancement.*—In 1959, Voisin showed that the mortality from runting syndrome in newborn mice could be favorably altered by the administration of antisera directed against the histocompatibility antigens of the recipient. This principle was extended to the mouse parent to $F_1$ hybrid model (A into $AB_{F_1}$). Anti-B antiserum was found to reduce mortality when A spleen cells were injected into $AB_{F_1}$ hybrid recipients (25). Similar results were obtained by Batchelor & Howard (26). It is important to realize, however, that the dose and schedule of administration are critical factors, and as yet these factors are not clearly understood. It is possible with this model to have antisera which do not give the expected beneficial effect but instead aggravate the GVH disease or have no apparent effect at all.

Buckley et al have used this approach to attempt immunologic reconsitution from a mother to her child with severe combined immunodeficiency disease (27). They gave maternal bone marrow and maternal serum to the recipient, reasoning that 15 previous pregnancies in this instance might provide a good antibody titer against paternal histocompatibility of the baby. Furthermore, the maternal serum was shown to block MLC reactions as well as to contain cytotoxic antibody. In addition, the maternal bone marrow was fractionated. It appeared that the child had partial immunologic reconstitution with obvious clinical improvement. No evidence of chimerism was found and the child died. A second child (28) was given human anti-HL-A-serum directed against HL-A 1 and 8 determinants which the child possessed but which were lacking in the father whose marrow was used as donor. The child died 12 weeks after being given $10^9$ bone marrow cells from the father but did not develop evidence of GVH disease. We have done a similar study using less than half this number of cells combined with frequent administration of antisera, and in our case we witnessed a devastating GVH reaction culminating in death 23 days later.

In retrospect, the interpretation of these observations is somewhat difficult. They do suggest, however, that certain antisera may suppress the capacity of donor lymphoid cells to establish GVH reactions, but when they do, there may be a danger that they will prevent or interfere with acceptance of the marrow graft itself. Other antisera against the HL-A antigens do not interfere with GVH. The question is whether in our latter case the antisera may have aggravated the GVH.

It is not yet clear either in experimental animals or man just what the optimal conditions are, and how one can predict the influence of such an approach. Studies of our successful human transplant patients (29) and experimental animals (30) suggest that an active serum factor, probably antibody, accompanies a chimerism. These patients, for example, possess lymphocytes which will destroy their own fibroblasts in vitro. Perhaps the reason these lymphocytes do not detroy host fibroblasts in vivo is that the patients also possess blocking factors or antibodies capable of interfering with this cytotoxicity. This model of GVH modification (immunodeviation) continues to be attractive but requires further experimental analysis.

*Suppression of bacterial flora.*—The pathogenesis of GVH remains uncertain but the cause of death in most instances is probably directly related to infection. Keast (31) working with rats and Jones et al working with mice (32) have increased the survival rate from GVH by reducing the host's bacterial flora. Jones was able to bridge major histocompatibility barriers with only mild GVH reactions and reduced mortality by using germ-free mice. However, others have had less success in their experiments with GVH in germ-free animals. Our experiences have been less rewarding but may relate to failure completely to eliminate the bacterial flora and other potential pathogens, even though we were unable to culture bacteria from gut, urinary tract, skin, or oral pharynx at the time of marrow transplantation.

A second problem with infection in these patients relates in a very interesting way to the host-parasite interaction. Two of our patients have died with unrecognized *Pneumocystis carinii* infection. Because patients with combined immunodeficiency disease lack capacity to react against microorganisms, they may have infection which is relatively occult and thus fail to show the classical manifestations of disease which result from the host's inflammatory responses to the infecting organism that are consequent to immunologic phenomena. At the time that immunologic vigor is developing after reconstitution, those patients previously infected but unable to express disease begin to manifest disease consequent to their newly acquired immunologic capacity, mounting an immunologic inflammatory reaction against the pathogen, which may be fatal.

Both of these aspects of GVH present major problems at the bedside. Our continued investigation in experimental animals and man will surely improve our approach and make marrow transplantation possible across major histocompatibility barriers.

*Clonal destruction.*—A more recent approach to circumventing GVH has been to attempt specifically to eliminate that clone of immunocompetent T-cells capable of reacting against the host, while leaving other immunocompetent clones of cells intact.

The first of these approaches is that used by Santos (33). In his experiments with mice and rats he induced a specific immune response to an immunologically competent recipient by injecting the potential donor cells into the recipient. When the recipient developed an immune response to the histocompatibility antigens of the donor, he gave massive doses of the cytotoxic agent cyclophosphamide. In this way he was able specifically to abrogate the immune response of the recipient to allotransplants from the donor. Application of this approach to marrow transplants has been accomplished in two ways. Donor cells are exposed in vitro to mitocycin-treated cells of the recipient. The donor cells, so activated, are then exposed to BUdR and light, specifically to kill those cells which had responded to the antigenic stimulus and had incorporated the BUdR (34). A similar approach has been tried with a hot pulse of tritiated thymidine (35). It was anticipated that these in vitro exposures would specifically eliminate those cells from the donor cell inoculum that could react against the host and initiate a GVH reaction. Thus far, this approach, perhaps not exhaustively tested, has been disappointing and at least two fatal GVH reactions have occurred.

Since these initial efforts much has been learned, and in spite of the difficulties encountered the approach to an otherwise fatal disease continues to be a challenge with promise of solution.

In addition to the use of marrow transplantation to correct severe dual system immunodeficiency, this approach has been employed with success in the correction of the Wiskott-Aldrich syndrome (36), idiopathic aregenerative anemias, pancytopenias, and radiation- or drug-induced bone marrow failure (37, 38). Here again the matched sibling donor is of greatest importance. Without a matched sibling donor fatal GVH disease has been the rule. In these cases GVH disease may be increased by cytotoxic and antimetabolite drugs required to facilitate acceptance of the foreign marrow graft in the immunocompetent recipient. Storb et al, working with dogs, discovered that when heavily irradiated or cyclophosphamide-treated recipients were given marrow transplants and the marrow transplants were accompanied by continuous treatment with methotrexate, the GVH was often abrogated (39, 40). Experiments in mice showed that cytotoxic drugs enhance susceptibility to GVH disease (41). Furthermore, fatal GVH reactions have occurred in humans given bone marrow transplants after treatment with cytotoxic drugs, even though a perfect match at both the HL-A and MLC loci has been achieved. The use of methotrexate to depress these threatening GVH reactions has been most encouraging.

Enthusiasm for the use of marrow transplantation as an adjunct in the treatment of leukemia has been diminished by experiences indicating that the marrow transplantation does not prevent recurrences of the leukemia and that the cells of the marrow transplant itself may "catch" the leukemic process (42, 43).

*Thymus transplantation.*—A second group of infants, with a congenital absence of the thymus, the DiGeorge syndrome, have also experienced successful immunologic reconstitution. These children often present soon after birth with hypocalcemia, typical facial anomalies, congenital heart disease, and varying degrees of impaired cellular immunity. It must be stressed, however, that some children with the clinical features of DiGeorge syndrome may have a small remnant of functioning thymus remaining (41, 44, 45). Some patients with all the other stigmata of the disease may have a normal thymus and full immunologic vigor. This makes the analysis of treatment in these children somewhat difficult. Nevertheless, there are 4 cases of thymus transplants associated with immunologic reconstitution in the DiGeorge syndrome (46–49). One very interesting observation in some of these cases is the rapidity with which the lymphocyte response to in vitro stimulation by phytohemagglutinin (PHA) returned. In two other patients without the classical DiGeorge syndrome, but with deficiencies in cellular immunity, the lymphocyte responses to PHA were reported to return as early as 6 and 48 hrs after thymus transplantation (50, 51). Such is not the case in the neonatally thymectomized mouse who is given a thymus graft. This rapid reconstitution may imply that the thymus graft may be providing a humoral factor which expands immunoincompetent postthymic cells already present in the patient. An alternative interpretation is that the deficiency is only partial in these cases and that unexplained fluctuation of the partial deficiency accounts for the apparently rapid reconstitution observed.

Biggar et al have recently shown in mice that the fetal thymus taken at 12–14 days embryonation (wet epithelial membrane) is capable of withstanding transplantation and will grow normally under the renal capsule of a syngenic host (52). When embryonic thymuses are grafted into neonatally thymectomized mice in both syngenic relationships and across major histocompatibility barriers, immunologic reconstitution is achieved. Further, a greater number of mice are immunologically reconstituted with these embryonic epithelial grafts across major barriers than when lymphoid thymuses of older donors are used. Furthermore, unlike adult grafts, the fetal thymus grafts do not induce a GVH reaction in these genetically susceptible neonatally thymectomized mice (53). Histocompatibility between the thymus and recipient is important, for we believe that as the immunologic capacity of the recipient is developed following thymus transplantation, the host's own immunocompetent T-cells may recognize the incompatible thymus graft as being foreign and reject it. Stutman et al have presented evidence of this in mice (54, 55). If the thymus donor and recipient are closely matched, rejection of thymus graft should not occur. Thymus grafts have also been used to treat patients with mucocutaneous candidiasis (56) and disseminated vaccinia gangrenosa with apparent beneficial effects. The exact immune defect in these patients, however, remains unclear and the role that the thymus transplants have played in reversing these infections is not at all clear.

*Transfer factor.*—Transfer factor, first described by Lawrence almost 20 years ago and as yet undefined at the molecular level, is a most fascinating, challenging, and controversial immunological phenomenon (57). This dialyzable substance obtained from leukocytes appears to have a low molecular weight, somewhat less than 10,000 (58). It is not an immunoglobulin and resists inactivation by DNase, RNase, and trypsin (58). It seems to be as effective as sensitized leukocytes in transferring delayed hypersensitivity to nonsensitized humans. It has been shown in man to transfer many different forms of delayed allergy (59) and to transfer allograft immunity (60). It has been used to convert negative to positive lepromin reactions in patients with lepromatous leprosy (61), to treat many cases of chronic mucocutaneous candidiasis, and even to produce a molecular intervention in a genetically determined disease, the Wiskott-Aldrich syndrome (62–63). One of the greatest obstacles to achieving a molecular definition of transfer factor has been the failure to develop an experimental model for its analysis. To date, all experiments have had to be carried out in man—a tedious and difficult assignment.

*Leukocyte transfusions.*—Another potential method of immunologic reconstitution has recently been presented by Lim et al (64). They infused buffy coat obtained from whole blood of lepromin-positive or lepromin-negative donors to treat lepromatous and mixed forms of leprosy. In their preliminary observations, dramatic healing of lesions, abrogation of the characteristic inflammation, and reduction of bacterial numbers has apparently been accomplished. These studies may represent another form of cellular engineering where a reaction induced by allogeneic leukocytes has the potential of activating the host's own biologic amplification mechanisms, which contribute resistance to infecting organisms and modulation of disease. Since they are so important, these observations deserve further controlled evaluation.

## SUMMARY

The preceding discussion describes only the first steps taken toward achieving the many potential forms of cellular and molecular engineering of immunodeficiency and other human diseases. From the first efforts to correct severe combined immunodeficiency disease, it is clear that this form of cellular engineering can be used to correct three genetically determined diseases: the X-linked and autosomal recessive forms of combined immunodeficiency diseases and the Wiskott-Aldrich syndrome. We have reversed an immunologically induced pancytopenia and it appears that acquired idiopathic pancytopenia can also be corrected by BMT.

Many of the diseases described above are relatively rare, but extremely important both for the critical questions which they pose and the opportunity they afford for analysis of this newer form of therapy, cellular and molecular engineering. Indeed, being able to switch blood types, if we could do it reproducibly and across all histocompatibility barriers, might give us an approach to many diseases in addition to the uncommon primary immunodeficiencies for which the methodologies have been described and will be perfected. Immunologic intervention and immunodeviation for tissue and cellular transplantation have advanced far enough so that we can be certain we are on the threshold of an era of medical research, and ultimately of treatment of disease in which biologic engineering will be a major approach.

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# VIRAL MYOCARDITIS AND ITS SEQUELAE

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Clinical evidence suggesting involvement of the heart has been reported for essentially all known viral infections (1). Histopathologic myocardial lesions have been identified in association with most viral infections (2, 3). The etiology of lesions demonstrated at postmortem examination after illness associated with hypoxia, electrolyte imbalance, secondary infections, or administration of catecholamines, however, is often uncertain, especially when the lesions are focal. The only viruses which have been repeatedly isolated from human hearts are Coxsackie, ECHO, and poliomyelitis. The former two have been firmly established as causal agents of acute myocarditis in the adult (4–6) and are generally considered to account for most clinical cases of viral myocarditis.

The clinical aspects of acute myocarditis associated with known viral syndromes have been reviewed recently (7, 8). The present review will focus upon proven viral myocarditis and selectively discuss certain manifestations, primarily in adults. Special consideration will be given to recent experimental evidence suggesting that there may be late cardiac sequelae, and raising the possibility that some cases of cardiomyopathy may represent endstages of viral myocarditis.

Our knowledge of the true incidence and natural history of viral myocarditis in man, possible differences between viruses, and the effects of interventions of possible therapeutic value remains fragmentary because the etiologic diagnosis of viral myocarditis is difficult and often impossible, even in fatal cases. Both alertness of the physician to the possibility of a viral infection early in the course of illness and access to a viral laboratory are necessary to establish specific viral etiology in a given case. Isolation of a virus from throat washings, feces, or pericardial fluid is generally possible only during the first few days of illness (1). Isolation of virus from the myocardium along with histologic study has been made possible by newer techniques of myocardial biopsy (9, 10). The presence of virus in myocardium has also been demonstrated by means of electron microscopy (11) and immunofluorescent techniques (12). Serological methods of diagnosis, which have become more generally available, permit the identification of a specific recent infection, although the attribution of a myocardial lesion to the identified virus remains circumstantial. A fourfold increase in titer of virus-neutralizing, complement-fixing, or hemagglutination-inhibiting antibody is indicative of a recent infection. Such studies require a high index of suspicion, leading to early and serial sampling of blood, and a laboratory willing and able to test sera against a large number of possible viruses. In the majority of cases of so-called viral myocarditis, the etiologic attribution is based upon clinical data alone or, in fatal cases, upon histological changes in the myocardium. The latter, however, are nonspecific, and other infectious and toxic forms of myocarditis are often difficult to exclude. Myocarditis is rarely associated with the easily recognized clinical syndromes of the viral diseases of childhood and then tends to be benign (8).

## CARDIAC ARRHYTHMIAS AND CONDUCTION DISTURBANCES

Although it has long been known that viral infections, among other inflammatory lesions of the myocardium, may give rise to cardiac arrhythmias as well as result in varying degrees of heart block, recent advances in electrocardiographic surveillance of cardiac patients as well as in therapy have increased our appreciation of these complications of viral myocarditis. Atrial premature beats and tachyarrhythmias appear to be remarkably infrequent, although they have been described in Coxsackie B (13) and ECHO 9 infections (14), in varicella myocarditis (15), and in infectious hepatitis (16). Ventricular premature beats, on the other hand, are not infrequent (5, 13). Ventricular tachycardia and fibrillation have been documented in association with Coxsackie B viral myocarditis (5, 13) and ECHO type 9 myocarditis (17).

All degrees of heart block have been observed in association with viral myocarditis. Both partial and complete heart block have been described in rubella (18), including a case of permanent complete heart block (19), mumps (20), including a case of Adams-Stokes syndrome treated successfully with a pacemaker (21), infectious mononucleosis (22), and influenza (23). Partial and complete heart block as well as bundle branch block, usually transient, have been observed in Coxsackie B myocarditis (5, 13). Monif and associates (17) reported the case of a 34-year-old dentist with acute onset of atrioventricular dissociation attributed to acute myocardial infarction who died within 24 hr of hospitalization after persistent ventricular fibrillation. Autopsy revealed diffuse inflammation and necrosis in the myocardium from which ECHO type 9 virus was isolated.

Among 65 cases of chronic heart block subjected to clinicopathologic analysis by Harris and associates (24), there were two cases of active nonspecific myocarditis with history of recurrent syncopal attacks of several months' duration. Active myocarditis involved both bundle branches but the A-V nodes were intact.

## SUDDEN DEATH

Although sudden, unexpected death in the United States is generally attributed to ischemic heart disease—often on the basis of coroner's certification or gross examination of the heart only—a small but consistent percentage of sudden death is proven to be owing to inflammatory heart disease. Thus, Kuller, Lilienfeld & Fisher's autopsy series (25) of 72 cases of sudden, unexpected death included the case of a 20-year-old woman who died suddenly at work, and the review of 275 sudden deaths in adults between the ages of 20 and 45 by Luke & Helpern (26) yielded one case of myocarditis. In Denmark, Gormsen (27) analyzed 1378 cases of sudden death and found 17 instances of myocarditis. Among 58 cases in which death remained obscure at gross autopsy, diffuse interstitial myocarditis was evident in 11. Corby (28) reported 32 cases of sudden, obscure death associated with focal myocarditis, representing 0.35% of medico-legal autopsies performed in the London area.

A study reported by Stevens & Underwood Ground (29) provides interesting data with regard to prevalence of asymptomatic myocarditis, and illustrates the difficulty of attributing death to focal myocarditis. Searching for evidence of sudden incapacitation, these authors examined multiple blocks of myocardial tissue in 263 pilots killed instantaneously in aircraft accidents and found six cases of isolated focal myocarditis. However, in no case did the evidence suggest that heart disease had incapacitated the pilot. Reviewing the literature, they found one case in which an aircraft accident had been attributed to myocarditis in a pilot.

## CHRONIC MYOCARDITIS

In 1806 Corvisart (30) asked: "Is the inflammation of the heart always very sharp and acute, or does it not sometimes affect an insidious, hidden progress, and which it appears, if not impossible, at least very difficult to distinguish?" While a definitive answer can still not be given with respect to viral myocarditis, evidence is accumulating that, in some instances, viral infection may result in subacute and chronic myocarditis, involving conductive system and valves, and that chronic fibrosis and cardiomyopathy may result.

Boikan in 1931 (31) applied the term "myocarditis perniciosa" to a case of interstitial myocarditis of at least four months' duration. In 1960, Kline & Saphir (32) collected from the literature 23 cases of chronic myocarditis characterized by progressive myocardial failure leading to death in months to years. Death had been unexpected in 16 of these. Kline & Saphir then reviewed their autopsy series of 225 cases of myocarditis, diagnosed on the basis of multiple cardiac sections from 2652 autopsies. Six cases were reclassified as chronic pernicious myocarditis on the basis of their protracted and relentless downhill course, over six weeks to six years, interrupted by remissions and exacerbations with progressive myocardial failure. All hearts were enlarged, without endocardial or pericardial changes. Microscopically, there was interstitial myocarditis with replacement fibrosis. Two of the patients had been misdiagnosed as mitral stenosis on the basis of apical diastolic murmurs, and one died after cardiotomy. While no cause was established for any of these cases, it must be pointed out that they preceded the era of availability of viral studies. Chronic inflammation may selectively involve the conducting system (33). Harris, Siew & Lev (34) reported a case of organizing myocarditis associated with partial destruction of both bundle branches, as well as acute inflammation of the A-V bundle and both bundle branches. Clinically, the process was documented over two years. The ECGs, correlating well with the pathologic findings, sequentially showed left posterior hemiblock, complete right bundle branch block and 2:1 A-V heart block, and complete A-V block due to trifascicular bundle branch block. It is unknown how often fibrosis of the conducting system may represent a late sequel of myocarditis, as suggested by Hudson (3). The case of permanent heart block following measles has already been cited (19).

A few follow-up studies of clinically acceptable cases of acute myocarditis reveal late manifestations which may or may not be related to earlier viral infections. Levander-Lindgren (35) reevaluated the cardiac status of 174 patients with myocarditis after a mean follow-up of seven years. Of 86 patients whose myocarditis had been isolated or associated with nonstreptococcal upper respiratory or other infectious disease, 38 still had cardiac symptoms, and in 25 the ECG was abnormal. There were three late cardiac deaths. Fifty-one of these patients were given an exercise tolerance test: 15 showed an abnormal exercise ECG, and the working capacity was low in 12. In seven patients the heart was still enlarged roentgenographically. Exertional dyspnea, easy fatigability, palpitations, and precordial discomfort were prominent residual symptoms. Physical examination was usually normal. Whereas most ECGs showed improvement, residual ST and T changes were not infrequent, and were often enhanced or brought out by exercise. It must be stressed that although undoubtedly some of these patients had viral myocarditis, none was proven. In the absence of a control series, the role of postinfectious asthenia or other disease developing concurrently is unknown (36). A five-year follow-up study by Bengtsson & Lamberger (37) of 90 patients with clinical acute myocarditis, associated with streptococcal disease in half the cases, revealed persistent cardiac symptoms in 20% and abnormal resting ECGs in 19%. Again, exercise ECGs were abnormal more often, namely in 30%, and the physical working capacity was reduced in 40%.

More recently, Bergstrom and collaborators (38) followed 15 patients after acute myopericarditis. Of six patients who had proven viral myocarditis (one infectious mononucleosis, two vaccinia, two Coxsackie B, and one ECHO), only two patients had residual symptoms at 46 and 40 months respectively, but these and one other had abnormal resting and exercising ECGs. Two others had abnormal standing ECGs. Working capacity and heart volume, however, were generally within normal limits. The authors concluded that persistent ECG change alone may be of no clinical significance.

Helin and associates (39) reexamined 12 patients at an average of seven months after acute myocarditis observed during a Coxsackie B5 epidemic. All were free of symptoms and had normal heart size; only one had an abnormal resting ECG. Most of the 22 patients with proven Coxsackie myocarditis reported by Sainani and associates (13) recovered within a few weeks to months. Five patients, however, had persistent signs of heart failure, as well as ECG abnormalities. In Smith's series of 42 adult cases of Coxsackie B myopericarditis (5), 82% made a complete clinical recovery, although recovery took three months or longer in 12 patients. Seven patients had one or more recurrences of cardiac illness, fatal in two. In six patients ECG abnormalities persisted for one half to six years, and three patients had residual cardiomegaly. Thus, there is at least circumstantial evidence that viral myocarditis may be subacute, possibly chronic.

Such observations have led to the hypothesis that some cases of chronic idiopathic cardiomyopathy may be the result of earlier viral myocarditis. This hypothesis has been strengthened by the detection, by means of fluorescent antibody techniques, of viral antigens in the myocardium of some cases of cardiomyopathy (40). The demonstration by Burch and associates (12, 41) of Coxsackie virus antigen in mitral and aortic valves of some patients with cardiomyopathy has even given rise to speculation that chronic valvular heart disease may be a sequel of viral endocarditis.

An unusual late complication of rubella myocarditis was described by van der Horst & Gotsman (42). A three-year-old boy had an apical left ventricular aneurysm in addition to a patent ductus arteriosus and stenosis of peripheral pulmonary arteries.

It remained, however, for Wilson and co-workers (43) to furnish experimental proof that viral myocarditis may be chronic and result in myocardial fibrosis. Experimental myocarditis in mice inoculated with Coxsackie B3 virus resulted in acute and chronic inflammation, followed by progressive myocardial fibrosis. Chronic lesions were evident in 53 of 70 animals. Inasmuch as virus could not be isolated from the myocardium beyond the sixth day after inoculation, it was postulated that the lesions represented an immune response, either to persistent viral antigen or to antigen newly formed from necrotic myocardium.

## MISCELLANEOUS EXPERIMENTAL STUDIES

In addition to the studies already cited, several other recent investigations are worthy of mention. Physical exercise was not only shown to increase the replication of Coxsackie B3 virus in the myocardium, but also resulted in an increased severity of myocardial necrosis and inflammation leading to congestive heart failure and death (44). Recently, another condition imposing a volume load upon the circulation was shown to enhance an experimental viral myocarditis. Farber & Glasgow (45) found that whereas replication of encephalomyocarditis virus in the brain of mice was unaffected by pregnancy, it was increased in the heart of pregnant mice. Pregnancy was associated with increased mortality and with myocarditis, as well as hepatic congestion, which were not observed in the infected but nonpregnant controls. Abnormal intraventricular conduction was also demonstrated. The hormonal changes of pregnancy, of course, could also have played a role via increased secretion of corticosterone, known to enhance experimental viral myocarditis (46). This study may have relevance to postpartum cardiomyopathy in humans, a still poorly understood entity which is increasingly being suspected to be, at least in part, of viral etiology (13).

It is of interest that although Pearce (47) had elegantly demonstrated that interventions leading to hypoxia increase the severity of experimental myocarditis, a recent study by Orsi and collaborators (48) revealed that exposure to hyperbaric oxygen also increases multiplication of Coxsackie B1 virus in the heart of infected mice and leads to greater mortality.

In a model of persistent gestational infection of chickens with mumps virus, St. Geme and associates (49) demonstrated transient fetal myocarditis, followed by persistent fibroelastosis.

## CONCLUDING REMARKS

Although a relatively rare form of heart disease, at least in the United States, and generally acute and benign, viral myocarditis may be severe, cause congestive heart failure, or, strategically placed, lead to life-threatening arrhythmias or conduction defects, and may result in a chronic state of myocardial inflammation. It is quite likely that viral myocarditis may be responsible for at least some cases of chronic cardiomyopathy. Sudden, unexpected death may be caused by viral myocarditis.

As diagnostic methods become more refined and more available, definitive studies of the natural history of viral myocarditis should become possible, especially during epidemics and utilizing interinstitutional collaboration. Controlled studies of the effects of therapeutic measures are needed. Widely available intensive and coronary care units should be used to give patients with myocarditis and cardiac arrhythmias or conduction disturbances appropriate surveillance.

The occasional chronic sequelae and rare fatal outcome of viral myocarditis constitute an argument for the search for specific treatment and prevention of viral disease. Thus, the cardiac complications of poliomyelitis (7, 8, 23) have largely been eliminated by the successful primary prevention of this disease.

A number of experimental models of viral heart disease are now available, and can be used to shed light upon the as yet unknown mechanism by which viruses produce myocardial disease.

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# METHADONE TREATMENT OF HEROIN ADDICTION 7068

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## Introduction

It is too early to expect or to provide a definitive assessment of the role of methadone in the rehabilitation of narcotic addicts. It is thought that some 60,000 persons are now in treatment from a reservoir variously estimated as 250,000 or 650,000. Our current opinion is that programs which offer a wide range of services, and which use methadone in support of their operations, can be useful for some 40 to 60% of addicts who volunteer for treatment, and can aid them in achieving a socially desirable change in life style. Physicians should nevertheless be aware of some of the current problems in evaluation in order to appreciate issues that may arise as programs and facilities proliferate.

Confusion of goals, complexity of the range of problems being treated, clashes of values as to the desired treatment outcome, different conceptions of the behavior entailed in addiction, and the consequent inevitable stereotyping and politicization—not only of drugs and the people who use them, but of the appropriate social response to them—are factors complicating sound medical and scientific evaluations. The many players on the stage—the police, community and neighborhood leaders, rehabilitation workers, physicians, ex-addicts, the pharmaceutical industry, the mushrooming private corporations selling packaged services ranging from therapy to the various techniques for urinalysis, various power groups in government from the executive to Congress, to agencies in the states and cities—hardly provide a scene which can be readily understood.

Attempts to control the giving and getting of psychoactive drugs will always involve social and biomedical issues, and evoke emotional response. To many, addiction is a frightening disorder, strikingly demonstrating man's capacity to be enslaved to a habit—a habit requiring the consumption of a substance which is sometimes difficult to obtain. Theories of addiction and cure have partially determined social responses. Thus, the use of narcotics may be viewed as a pattern of behavior which in some instances is controlled (as with weekly or monthly opium smoking), but which in other instances is not controlled (as in the case of contemporary "junkies," or the middle-class ladies who, at the turn of the century, consumed opium-tinctured medicines to ward off withdrawal). With such a view, treatment could be directed toward achieving control over whatever drug was taken and the only population at risk would be those who could not exert such self-control over their drug consumption. If the inability to exert control over repetitive drug use is a flaw of the will, but one easily corrected by medical treatment, then all who are vulnerable should be expected to avail themselves of such treatment; such weakness or avoidance of treatment could be viewed as a willful flaunting of society's preferred behaviors, and criminal sanctions might well be in order.

Musto (1) has documented that at the time of the drafting of the Harrison Narcotic Act of 1914, American medicine believed that there was a quick medicinal "cure" for addictions; obviously anyone who could avail himself of some permanently effective medication ought to do so, and drastic laws to do away with the rest of the problem would be justified. If human folly is exploited by the cupidity of entrepreneurs, then they will be the target (pushers, Mafia, etc) of society's response. If physicians generate addiction as a byproduct of their medical and surgical treatments, then it is their mode of prescribing in the management of patients which would require careful attention. If the person to become addicted could be predicted—the legend of the addictive personality—then individuals could be assigned to their recreational drugs of choice—obviously (in this context) a "pipe" dream!

The majority of those tolerant to high doses of opiates, physically dependent and subject to withdrawal symptoms, may be readily detoxified, but they readily return to their habits once they leave a treatment system. These individuals have "learned" in experiencing the effects of the narcotic to avoid the daily menu of unpleasantries which can create tension; social relations are frequently involved in the task of procuring drugs and a life style becomes established. It is an adaptation of surprising durability. For many detoxified patients it takes but the sight of an old friend from the addicted phase to create a powerful psychological impetus to relapse. Addiction involves learning to exploit the effects of self-administration of a chemical which has tranquilizing, antianxiety, and sometimes euphorigenic effects, and learning to depend on its intake and the *circumstances* of its intake as a central style of life. An old habit that has immediate utility (despite large subsequent cost) is not readily forgotten.

Wikler has described a conditioning theory of addiction (2). Dole feels that the effects of the opiate drug itself may generate destructive patterns of behavior. In his view, a "hunger" is created (or the individual is vulnerable to this "hunger" for genetic or other reasons) and one treats the biological source of the hunger—blocking it with methadone—to shift the patterns of desired behavior (3, 4). There is extensive research on the biochemistry of so-called pleasure centers and brain mechanisms involved in motivation, but there is little clearcut evidence that would sharply specify this "hunger" theory as yet.

There are different populations involved in addiction at different times in history. Our recent epidemic of drug interest involves widely different sectors of society. Addiction to narcotics, as a stereotype of Black and Puerto Rican ghettos, is not tenable when white and middle-class people are—as they were at the turn of the century—now involved. The health professions have always been preferentially at risk—probably simply because of the availability of drugs. It follows, and our observations confirm, that different treatments are useful at different times for different subgroups, depending on pattern of use, age, subculture, etc. The ultimate goal, of course, is prevention—however one views the nature of the relapsing disorder.

The bulk of evidence, we believe, indicates that addiction is a disorder profoundly reinforced in its *initiation* by social-psychological as well as pharmacological factors. "Friends" group together and supply each other to be different, "cool," and daring. Addiction is then sustained by social and psychological factors in which the reinforcing value of narcotic drugs plays an important, but not an essential, role. Whether tranquilizing and antianxiety drug effects are contributory motives in sustaining addiction is as yet unclear; but we do know that euphorigenic effects decrease and in some instances disappear entirely as the dependence grows older. We do not think that physical dependence itself is sufficient to generate the profoundly self-destructive behaviors characteristic of the modal pattern of narcotics addiction with which the public is most concerned. Many individuals are made physically dependent in the course of medical treatment; they experience mild withdrawal but do not engage in sequences of self-administration of drugs, and the opiates do not become a central part of their existence, as they do for the prototypical addict.

Social and psychological factors have been stressed because they are clearly the key elements in the treatment of addictions. The addict who comes for treatment shifts the definition of drug-taking from hustling for drugs in the streets to seeking administration—or prohibition—of drug-taking under the direction of medical authority and management. This, of course, means that the community and medical authority must be ready and willing to receive and reinforce such motivation, and the use of opiates is meant to relieve some of the destructive self-medicating behaviors of the addict. It is quite possible to dispense opiates without this aim in mind, but simply dispensing drugs, in our view, does not constitute a fully adequate concept of treatment.

It has been noted (3–5, 8) that if the goal of treatment is the production of an individual who, while "addicted"—i.e., dependent on medically procured narcotics—is nevertheless productive and socially responsible, substantial progress is possible. If the aim of treatment is to stop criminal behavior, this is obviously not entirely possible with any group of individuals, whatever means are employed. If the aim is to produce drug-free individuals who are socially responsible, as is the case in the therapeutic community paradigm, it is clear that, while some addicts respond excellently, about 12 out of 13 drop out. Accordingly, from a broad public health perspective, other approaches in addition to the drug-free communities are currently necessary as integral components of a broad scale attack on the problem of addiction.

In summary, in thinking about the efficacy and indications for methadone maintenance treatment, it is practically useful to emphasize that a significant factor in treatment is the shift in the definition of the *sponsorship* (and hence the purpose) of drug-taking, rather than an enigmatic power of a particular chemical. In brief, our task remains familiar to medical psychology—a sustained commitment to the social and psychological rehabilitation of individuals who have learned self-destructive patterns of behavior.

## PHARMACOLOGICAL AND MEDICAL ASPECTS OF METHADONE

Methadone (6), first synthesized by German chemists, is *d,l*-4,4-diphenyl-6-dimethylamino-3-heptanone; it owes its analgesic activity almost exclusively to the L isomer. Methadone has subjective effects similar to those of morphine, including euphorigenic effects in experienced users; it is equipotent in comparison with morphine, while heroin is approximately two to four times more potent than either (7). Ordinary analgesia follows the parenteral administration of 5 to 10 mg methadone. The duration of analgesia (3 or 4 hr) is similar to that of morphine, but the discomfort of withdrawal does not commence for 8–24 hr after methadone, while morphine "holds" for only 4–6 hr (7). Methadone differs sharply from morphine in that it is effective orally, and not only has a longer duration of action in suppressing withdrawal, but a longer half-life. These latter facts determine its usefulness in treating heroin addicts, for less frequent administration provides practical conveniences and oral dosage obviates the noxious side effects of intravenous "needling."

Methadone is a respiratory depressant; like other opioids it has antitussive properties. It produces mild hyperglycemia and hypothermia, and in general its neurophysiologic effects are similar to those of morphine. In man, methadone produces constipation, probably because it markedly inhibits intestinal tone. Methadone can cause biliary tract spasm, but it does not have major effects on the pregnant uterus. Tolerance to miotic effects of methadone—unlike those of morphine—develops rapidly. There are not prominent cardiovascular effects following methadone administration in man, and after intravenous administration, there is no "pins and needles" sensation as is induced by morphine (and attributed to histamine release).

Methadone, unlike morphine, is well absorbed from the stomach and large concentrations appear in the plasma within minutes after oral administration. The drug leaves the blood and enters tissues throughout the body with only a small amount traversing the blood-brain barrier; methadone appears to be bound to tissue protein; biotransformation in the liver is probably through N-methylation. Less than 10% of the drug can be recovered unchanged. The remaining 90% appears in urine and feces as degradation products whose character remains unknown. The clinical pharmacology of the drug is still under investigation.

Methadone has a number of side effects including sedation, constipation, excessive sweating, urinary retention, and change in the libido—usually a decrease but occasionally an increase. Rarely pruritus, nausea, delirium, or urticaria have been reported. Methadone, as with other drugs cross-tolerant with morphine, has a major potential for abuse; primary methadone addicts existed long before its current widespread medical use. Tolerance to the drug develops rapidly, except for its constipating effects. The withdrawal syndrome in the dependent person varies greatly in duration and severity. Although ordinary textbook descriptions state that the methadone withdrawal syndrome is, in comparison to the morphine withdrawal syndrome, more prolonged and less severe, many observers report that in some subjects the reverse is true. Some—not all—patients on oral methadone may occasionally wait for some withdrawal and then take their dosage to experience some "kick," but this is not an efficient way to experience a subjective effect and is a pattern of use rarely sustained.

Methadone is an effective drug in treating the withdrawal syndrome encountered in hospitals or in private practice. Withdrawal can in most instances be accomplished over a 7-day period, with initial total oral daily dose rarely needing to exceed 50 mg on the starting day of the detoxification schedule, with 5–10 mg decrements on each succeeding day.

Medical or surgical crises in methadone-maintained patients are best managed by continuing methadone at usual maintenance levels with once a day oral administration. If parenteral administration is necessary, 10 mg doses can be administered two or three times daily; rarely this parenteral dose level will need to be increased, but usually it will suffice regardless of the precrisis maintenance methadone dose. Analgesic needs in methadone-maintained patients require usual doses of demerol in addition to the maintenance level of methadone. Occasionally one encounters narcotics addicts who also abuse alcohol and barbiturates, and may exhibit, during medical or surgical crises, a withdrawal syndrome in which methadone in conservative doses can be used to treat the opiate withdrawal component, barbiturates in conservative doses can be used to treat the barbiturate withdrawl component, and diazepoxide or chlordiazepoxide—again in conservative doses—can be used to treat the alcohol withdrawal component. The principle is that each withdrawal component must be treated as if the others were not present. We recognize that theoretically the alcohol and barbiturate withdrawal component is treatable with one drug (say diazepoxide or chlordiazepoxide), but practically we feel that the regimen we describe should be adhered to until further experience is gained with this unusual problem.

Overdose syndromes related to narcotics must be treated with nalorphine and in the instance of overdose from methadone, observation must be extended because of the short duration of action of nalorphine in comparison to the length of action of methadone; i.e., repeated doses of nalorphine may be required in cases of methadone overdose. In the passively addicted newborn, withdrawal can be treated with paregoric, methadone (.1 mg/kg), or barbiturates.

## METHADONE TREATMENT

Dole & Nyswander (3–5, 8) pioneered the use of methadone in the treatment of heroin addicts. The daily oral administration of a "high dose" (about 100 mg) appeared to enable significant numbers of addicts to: (*a*) abolish or decrease their use of heroin; (*b*) abolish or decrease their criminal activity; and (*c*) increase the rates at which they sought and obtained legitimate employment. The technique initially employed was to hospitalize the addict and over a carefully monitored 6-week period to increase daily methadone dosage to levels ranging from 80 to 120 mg. Patients so treated did not report sedation, nor suffer from reduction in general alertness. Medical and psychological testing indicated that this regimen was safe. Patients reported a disappearance of "drug hunger," and were refractory to the effects of intravenously administered heroin. Once established, methadone "blockade" of the effects of heroin did not appear to require higher and higher doses of methadone.

Dole & Nyswander reasoned that they were in fact treating a metabolic defect. The "metaphor" justifying their medically supervised maintenance treatment (and serving to ward off moralistic attacks on keeping individuals addicted) was that of insulin requirement in the diabetic. Further, it was argued that in an epidemic of a germ-spread disease, medicine would intervene, if not to cure, at least to ameliorate consequences. So too with methadone and the addict.

In the Bernstein Institute of Beth Israel Medical Center in New York, the early studies were expanded with outpatient rather than inpatient initiation of treatment—now characteristic of most programs. Admission originally was confined to uncomplicated cases between the ages of 21 and 39. Patients had to have used heroin for at least 5 years, be neither psychotic nor alcoholic, medically ill nor mentally deficient, and have a history of previous treatment failures. Oral methadone was administered daily under direct observation, and later "carry out" privileges were selectively given to trustworthy patients. Most clinics now use the drug dissolved in fruit juice to control intake and discourage extraction for diversion to intravenous use; methadone "diskets" are marketed for similar purposes, but are not foolproof.

Results of these early studies were promising (9). Approximately two out of three patients were retained in treatment at the end of a 42-month study period in a patient population of 3372. On follow-up of those who left treatment, results differed depending on whether separation from the program was voluntary. Involuntary discharge was more likely to be associated with arrest, while voluntary discharges had a higher rate of entering other treatments, fewer arrests and, in general, a better outcome. Approximately one third of those leaving treatment voluntarily reentered the Dole-Nyswander program. Arrest rates present a problem in that in many "studies" they are based on self-reports unmatched by often equally unreliable police records. Nevertheless, in this study, they were the best available index of criminality, and showed substantial improvements over baseline values in 2560 patients studied over a 4-year period. Social adjustment as measured by return to school and legitimate employment showed similar gains. The findings also included negative results. Approximately 10% were noted to change drugs of abuse from heroin to alcohol, cocaine, barbiturates, or amphetamines, and many had to be discharged from treatment.

In 1968 the Methadone Maintenance Evaluation Committee (of the AMA) reported, "It does appear that those who remain in the program have, on the whole, become productive members of society in contrast to their previous experience, and have, to a large extent, become self-supporting and demonstrate less and less anti-social behavior (10)." The committee recommended further expansion of methadone programs.

An important element in the operation of methadone programs was the development of techniques for detecting a wide range of substances in urine. Thin layer chromatography (with prior extraction by organic solvents and ion exchange resins) initially was utilized and urine specimens were examined at least weekly for presence of morphine (heroin is detected as morphine in urine) and other drugs (usually stimulants and barbiturates). Urine monitoring offered an opportunity for more honesty between addict and physician than had existed heretofore. Positives meant that the patient needed help, while "clean urine" indicated increasing ability by the addict to control his behavior. Unreliable patient reports were no longer the sole basis for action.

The care with which the Dole-Nyswander program was conducted is not necessarily the pattern with which the hundreds of current programs throughout the country are operated (e.g. urine monitoring, if poorly used, can complicate and inhibit the growth of a real relationship between the addict and his counselor). The consequences of a urine report, indicating either that heroin or sedatives, or stimulants were taken, thus breaking "the doctor's orders," can mean a drastic change in the life of the patient—from being excluded from the program to being forced to change his daily habits and attend the clinic more frequently. The establishment of a punitive-legalistic relationship centered on urine results rather than a doctor-patient relationship occurs all too frequently.

The reliability of urine-monitoring techniques remains an unresolved issue. Technicians claim high reliability or sensitivity (referring at times to the absolute sensitivity of the detection system, at times to efficiency of extraction or minimal detectable amounts of drug in urine, often without regard to controlled dose and time studies). Every clinician has experience suggesting that "on line," in large urban programs, these techniques are frequently unreliable. The problems of control and reliability are quite similar to that noted in any large scale medical laboratory service. False positives and the mixing and misidentification of samples occur; quality control is required. Specificity of methods vary and naturally occurring urinary metabolites or therapeutic drugs must be considered. Urine tests are not only used for treatment decisions and initial diagnosis, but for epidemiology.

We simply are not at all, in fact, ready to make public policy or consequential decisions about communities and individuals without careful control over these problems which are familiar to every medical investigator and practitioner. Newer methods of immunoassay such as the free radical assay technique (FRAT) (11) and radioimmunoassay (12) are under study. Because there are different problems to be addressed—such as detection of the occasional user 24–48 hr after a single dose, and the chronic user (readily detectable with TLC)—a careful evaluation of these techniques is required and recently was begun.

Dole & Nyswander's work with over 3400 patients has been essentially corroborated in Washington, D.C. by Dupont (13), in Philadelphia by Wieland (14), by Jaffe et al in Chicago (15), Kleber at Yale, Knowles in St. Louis, Ramer in San Francisco, and others. Patients who remain in maintenance programs achieve a level of vocational and social stability to a degree never before observed in drug-abuse treatment. Contrasted with results of incarceration at Lexington

(95% recidivism), methadone-based treatment appears to offer substantial advantages.

Because large programs have accepted varieties of patients, e.g. behaviorally disturbed (who, by design, were excluded from the Dole-Nyswander program), their results may be somewhat poorer than those reported in the early days. It appears that retention rates after two years are closer to one out of two than to the two out of three figure reported in Dole's program.

There are widely different subpopulations of patients entering programs. A sizable subgroup, perhaps as large as 20%, appears to pursue a change in life style with a purpose strong enough to carry them through many adversities. The fact that addicts over 25 characterized the initial treatment groups led some to speculate that the costs of drug life were wearing down the experienced addict. The treatment of younger poly-drug users who "chip" (sniff or subcutaneously inject low grade heroin, and show little physical dependence) is of increasing interest. Another subgroup, substantial in size, appears to need to fail at treatment one or more times before reentering and becoming successful. In urban programs, a variety of subgroups can be identified, each with different paths.

Methadone maintenance has been and still is highly controversial. For many —physicians included—there was question of the efficacy of substituting one narcotic habit for another. Community objections, particularly among minority groups, have been vocal. There were initial, and in some cases, persistent fears that methadone was a form of social control or enslavement of the minorities. With the passage of time and the inspection of results, many have concluded that such programs are beneficial and have supported their expansion.

Factionalism in the field has chiefly, but not exclusively, been between those favoring methadone maintenance and those favoring therapeutic communities. Lennard et al (16) express the fear that adoption of methadone maintenance on a massive scale will inhibit the growth of the therapeutic communities and the drug-free movement. The option of medically available drug might indeed turn some to an "easier" road. But this fear—often advanced to pander to latent prejudices, or to serve political views of warring factions from police to therapeutic zealots—is not well substantiated. The fact is that the number of therapeutic communities has increased rapidly during the past few years in parallel with the growth and popularity of methadone maintenance.

Jaffe and the University of Chicago group successfully overcame the problem of factionalism by creating the multimodality concept in which all the available treatment methods, e.g. methadone maintenance, therapeutic communities, halfway houses, etc, were combined in one program with a central intake under a common administrative umbrella. The multimodality concept appears to have provided an excellent milieu with respect to research as work was carried out on the narcotic antagonist, cyclazocine, on methadyl acetate (a long-acting methadone congener), as well as the fundamentally important epidemiologic studies of Hughes et al (17).

The basic paradigm for drug services at present is composed of daily administration of oral methadone plus the so-called "ancillary" services consisting of vocational, legal, and social counseling. In most methadone maintenance programs, attendance in group therapy is optional. These groups tend to be of a confrontational nature: emphasis is placed on honesty and direct reporting of feeling states. The approach leads to intense emotional experiences, but many patients cannot adjust to such levels of feeling nor to the anxiety-provoking style of such groups.

A striking phenomenon in the addiction services field is the emergence of the ex-addict. These persons have taught many professionals the extent of commitment entailed in treatment, as well as some of the traits and meaning of the behaviors of drug-dependent individuals from minority groups. Ex-addicts have distinguished themselves in every phase of addiction services, e.g. in administration and creative program planning, but problems of their continuing professional role develop as treatment programs become institutionalized. For some workers it is difficult to shift to other jobs and a linkage to treatment through their jobs in programs may be an important anchor to avoid "crossing the line" and relapsing. Today, methadone maintenance programs throughout the country vary widely in the degree to which they are community based and controlled, and in the balance between professional and paraprofessional staff in the operation of clinics.

The safety of chronic methadone administration has not been seriously challenged. On clinical grounds, we have observed improvement in the altered liver function tests which characterize approximately 90% of patients on admission (18). Gearing has reported that death rates of patients who remain in treatment in methadone maintenance programs is similar to that seen in the general population, while patients who drop out of treatment have substantially higher death rates (31). Methadone maintenance, then, may be preventing the death of some who without treatment would die of overdose or medical complications of unsterile injections.

There are no known toxic effects of the long term use of heroin, and the so-called British system of heroin maintenance has been of interest. Recently, methadone has begun to be more frequently prescribed in Britain. Unlike the American system, opiates are generally self-administered and taken intravenously in outlying social centers (with "shooting rooms") or lavoratories, or at home, and drug is obtained, not at the clinics, but from designated pharmacies. The option to prescribe oral medication is not widely exercised. Expectations of patients and their physicians (supported by police and health bureaucracies) dictate such consequences to a degree; i.e. that all addicts must "shoot" and little can be done to avoid this. The major societal aim is to keep the price of illicit drugs down (and hence crime, and hopefully new addicts) by providing free drug and medical facilities. The spread of addiction noted in the late 1960s was traceable to physicians who were overprescribing and the British had therefore to limit the right to treat addicts with addictive drugs, and to control carefully the numbers of facilities in which this would take place. There are, in fact, many British "systems," some stressing rehabilitation and work as a condition for obtaining free drug, but all respecting the power of the addict's drug-seeking in playing a central role in their often disorganized lives. Heroin maintenance as a method of recruiting some subgroups into treatment has been proposed but as yet not sanctioned in the United States.

In recognition of the growing interest in oral methadone maintenance, the Food and Drug Administration issued guidelines on April 2, 1971 which paved the way for further extension of methadone maintenance services. The fear of diversion of methadone and the need to regulate the disposition and traffic of the drug may mandate stricter controls, in terms of limiting the amount of medication even trusted patients can take for home consumption (i.e. "take home" is a major avenue for diversion). Multiple registration of the same patient occurs. These problems may mandate excessive controls which challenge the confidentiality of records and the dignity and the motivation of the patient; the medical profession may have to accept constraints on the general use of methadone to simplify monitoring of supplies and avoid these excesses of control (19).

Methadone has been used to maintain pregnant women (20). Surprisingly, in our experience, newborns of methadone-maintained patients do not frequently exhibit withdrawal syndromes and usually do not need treatment with methadone or barbiturates. Pierson et al (21) have reported sudden deaths in infants born to mothers maintained on methadone, but whether methadone plays a role in such deaths is not clear. Many factors, such as malnutrition, and poor prenatal care, complicate the situation in pregnant addicts. A growing number of physicians feel that methadone has a place in the management of adolescent heroin addicts (22). Understandable apprehension concerning the use of methadone in this population must be balanced by considering the dilemma of physicians who have been confronted by 15-year-olds with a 3- to 6-year history of daily intravenous use of heroin.

Dole has recently described a program for utilizing methadone to treat a large number of young and older addicts who enter the corrections system in New York City (23). Dole states that approximately one half of the 34,000 addicts admitted annually undergo withdrawal symptoms. He attributes an improvement in general prison morale to the fact that the withdrawal syndrome is now adequately treated. He also feels that suicide rates were affected favorably by his program.

A major question raised by the maintenance of large numbers of patients on daily methadone relates to basic treatment goals. Dole states that addicts may require methadone for the balance of their lives. Experimental work done by Katon (24) and Chappel & Senay (25) suggests that abstinence is an achievable goal for at least some patients who have been maintained on methadone. The process of withdrawal to successful abstinence requires a much higher degree of attention and support than that required to maintain patients. Some initiate their own withdrawl; others determine the timing and occasion with the staff.

Goldstein (26), Jaffe et al (27), and others have demonstrated that program outcome is not significantly different in patients given high dose, i.e. 100–120 mg, vs patients given low dose, i.e. 40–50 mg. These findings call into question Dole's blockade theory. Clearly low-dose methadone is much more variable in the degree to which it induces cross-tolerance to heroin when compared to high-dose levels. Yet program outcome does not change as a function of dose. Patients on 50 mg given once daily may initially complain of discomfort at 16–18 hr after administration, but seem to adapt readily to the 24-hr regimen. Goldstein (28) and Senay & Wright (29) have discussed extensively whether the therapeutic effect achieved in methadone maintenance programs is best understood in pharmacologic, conditioning, or psychosocial terms. Is the drug the treatment, or does it facilitate the treatment?

## CONCLUSION

It is too soon, then, to evaluate firmly how the use of methadone may aid the rehabilitation of persons addicted to narcotics. At this writing, it appears that its use certainly will be important in the broad response to the narcotics problem, i.e. it probably will become a stepping stone for many addicts. For others, the problem of inducing the initiation of treatment may require different measures; heroin maintenance has been suggested largely to reach the population firmly committed to addiction who are not ready or willing to exert the motivation required by methadone programs in which patient status is almost exclusively voluntary. It will require a sanction not yet forthcoming by the professions and the public. Treatment of various subgroups (parolees, court referrals, "pseudo-junkies," etc) will require yet other responses. The use of longer-acting oral medications such as methadyl acetate (30) may ease some problems contingent on "carry out" medications. After all, the desirable goal is to provide the addict with treatment that turns him toward a life as free of clinical commitment as possible. The fear of diversion of methadone, of accidental overdose, may require technical improvements (stable isotope labeling of different sources of drug), as well as good discipline in clinics, to avoid overly coercive treatment programs.

It is clear that the proper use of methadone does provide a wedge into the problem of the destructive, repetitive use of drugs. Current consensus among professionals in the field of addiction services is that the appropriate use of methadone is safe and beneficial for the populations served, and success depends most critically on the manner in which relationships with drug-dependent persons are established and on the medical and rehabilitative commitment of the treatment system.

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# ESTIMATION OF GESTATIONAL AGE IN THE FETAL STATE

7069

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## INTRODUCTION

During the first half of 1972 the fertility rate in the United States was 72.7 births per 1000 women in the reproductive age group, lower than any rate previously recorded (1). In the immediate future, the expected trend for married women aged 18 to 24 is 2.1 births per woman. Whether this voluntary limitation in family size will continue is unknown, but certainly the emphasis on decreased quantity has led to heightened interest in improved quality of progeny. Although obstetricians and pediatricians have always been concerned about the long range impact of intrauterine illness, precise and feasible methods of fetal diagnosis have become available only quite recently (2–6).

Two of the most important decisions in fetal management involve determining the optimal time and method of delivery. This pertains especially to such complicating problems as maternal diabetes or hypertensive disease; retarded intrauterine growth; and erythroblastosis fetalis, where early delivery can reduce the risk of fetal death in utero. The obstetrician must decide whether continued intrauterine existence is more or less hazardous than extrauterine existence. In these complicated cases, length of gestation is often poorly documented. Since the risks of major respiratory and neurologic sequelae associated with premature delivery increase in proportion to the degree of prematurity, it is critical in the management of such cases to assess the extent of fetal maturity. This review is concerned principally with the present-day means of estimating the state of maturation of the unborn child.

## EVALUATION OF THE DURATION OF THE PREGNANCY

The normal fetus can be expected to have reached specific phases of maturation at given lengths of pregnancy. Therefore, knowledge of the length of gestation provides an important reference and the one most commonly used to estimate the status of fetal development. The date of conception may be identified if the patient has maintained a careful record of basal body temperatures. However, this is unusual. Estimation of the duration of the pregnancy on the basis of the date of the last menstrual period is still the most frequently employed technique and is probably 80% dependable (7).

By convention, obstetricians generally date the duration of the pregnancy in relationship to the onset of the last menstrual period. (The date may be "corrected" depending on clinical findings.) Embryologists and pathologists usually date pregnancy from the known or suspected time of conception. It is imperative to designate which is being used as the time of reference, for the two week discrepancy can mean a 20% difference in chances of survival. Throughout the remainder of this discussion, we will use the menstrual dates (or an extrapolation thereof) as the reference point in citing the duration of the pregnancy.

If there is doubt that a given menstrual date did precede conception by two weeks (questionable history, recent lactation, recent use of anovulatory drugs, etc), an accurate clinical assessment of the duration of the pregnancy becomes very important. The times that subjective symptoms (nausea, fetal movement) are first noted can be of some assistance, but the physical findings are more dependable. The earlier the patient presents, the greater the reliability of this assessment.

It is much easier to distinguish a 6, 10, or 14 week pregnancy by uterine palpation than it is to distinguish a 28, 32, or 36 week pregnancy (8). The fundal height is normally about 20 cm at 20 weeks and 30 cm at 30 weeks. However, the height or size of the fundus does not correlate well with the duration of the pregnancy after the 30th week. After this time, a number of variables (quantity of amniotic fluid; fetal size, position, and station; the presence or absence of uterine contraction; etc) appear to influence the measured height of the fundus more significantly. If the pregnant uterus seems disproportionately small-for-dates, one should consider the possibilities of incorrectly assigned dates or retardation of fetal growth (9). If the pregnant uterus seems disproportionately large, one should consider the possibilities of incorrectly assigned dates, the presence of more than one fetus, accelerated fetal growth, or excessive amniotic fluid.

It is also very helpful in assessing the duration of the pregnancy to determine the time when the fetal heart is first heard with the DeLee-Greenhill stethoscope. Usually this occurs at 18 to 20 weeks (10). On rare occasions it may be detected as early as 15 weeks, but it is unusual not to have detected it by the 22nd week. When there is question about the pregnancy duration, weekly examination for fetal audibility should be performed.

During early pregnancy, the maternal serum concentration of chorionic gonadotrophin rises sharply to a peak value and then gradually decreases to a stable value at 130 days, where it tends to remain throughout the rest of pregnancy (11). The approximate time of peak concentration can be detected with sequential determinations and may be equated with the seventh or eighth week of pregnancy. All of the above methods of assessing the duration of pregnancy have one important common feature: their accuracy is significantly enhanced by presentation of the patient early in pregnancy.

There are a number of maternal plasma constituents whose concentrations continue to rise during the course of pregnancy. These include placental sommatomammotropin (12), alkaline phosphatase (heat stable) (13), and cystine amino peptidase (14), as well as urinary estriol (15). However, there is too much variability in all of these factors for their analyses to be helpful in determining the length of the pregnancy.

## ASSESSMENT OF SPECIFIC FETAL SYSTEMS

### AMNIOTIC FLUID ANALYSIS

Although it is impossible at present to obtain blood or tissue directly from the fetus, material is obtainable from the amniotic fluid compartment. The fetus contributes a variety of fluids and cells to this space including fetal urine, lung fluid, and exfoliated epidermal cells. The analysis of these components has proven useful in determining fetal maturation. Distinctive changes are noted in all three around the 37th week, which is the stage in fetal lung maturation when the hazards of neonatal respiratory problems are minimal.

Although amniotic fluid is usually readily obtained by amniocentesis, the procedure is not without risk. Laceration of the placenta with fetal hemorrhage and needle trauma to fetal eyes, central nervous system, and abdominal viscera have all been reported (16–21). If the procedure is performed by an experienced operator and the customary precautions are taken, the risk of fetal complication must be no greater than 0.5%. Nevertheless, the potential value of the procedure must be weighed carefully against the hazards.

*Amniotic fluid cytology.*—Brosens & Gordon reported in 1965 (22) that near-term amniotic fluid could be identified by the presence of cells which stained orange when mixed with 0.1% Nile blue sulfate. These investigators subsequently noted that although most of the cells stained blue, the percentage of orange-staining cells tended to rise above 10% after the 36th week of gestation (23). A number of investigators have since confirmed that the presence of >10% orange-staining cells is compatible with fetal maturity (24–27). However, false negatives do occur in that percentages below 10% have been observed in 10 to 40% of "mature" pregnancies (28, 29). Therefore, the test is only helpful when a significant percentage of orange-staining cells is detected.

Most observers agree that adherent sebum accounts for the orange-staining features of these cells and that their increased number is a reflection of fetal sebaceous gland development and secretion (28, 30). [The presence of free orange-staining lipid globules and gross flecks of vernix caseosa in the amniotic fluid are also acceptable as evidence of maturity (25).]

Several investigators have found that the morphologic features of amniotic cells are more helpful in determining maturity than the lipid-staining characteristics (31–34). Bishop (35) and Huisjes (33) have noted a marked increase in the percentage of large anucleate polygonal cells present after the 36th week, which has been attributed to a sudden increase in the desquamation of fetal skin. Although these cells are probably the same ones that stain orange with Nile blue sulfate, this feature depends on the available neutral lipid. The incidence of false negative results appears to be less when cellular morphology is used as an index of maturity. The major advantage of the Nile blue sulfate staining method is its expediency.

*Amniotic fluid creatinine.*—As gestation progresses, fetal urine formation increases and has greater influence on the solute composition of the liquor amnii.

The fetus forms a hypotonic, protein-free urine (36), and the liquor amnii becomes progressively more hypotonic relative to maternal and fetal serum. After the 28th week of gestation the osmolality of amniotic fluid decreases from the isosmotic values of 280–285 to 230 mosm/kg$_{H_2O}$ (37), and the sodium concentration is similarly reduced from 139 to 120 meq/liter (38). There is considerable variation, but beyond 36 weeks gestation the osmolality of amniotic fluid is $\leq 255$ mosm/kg with few exceptions in normal pregnancies (39, 40). With hypoxia, the fluid osmolality remains elevated (40, 41).

The protein concentration in amniotic fluid is progressively reduced by virtue of the protein-free urine of fetal origin that is added to the sac. The total protein concentration falls from 300 mg% at 35 weeks to <200 mg% by 40 weeks in the absence of intrauterine hemolytic disease or other forms of hypoxia. Since bilirubin is bound to albumin, this bile pigment also shows progressive dilution with gestation, and the corrected absorbancy at 450 nm for bilirubin in aminiotic fluid falls from 0.060 (0.100 mg%) before 35 weeks to <0.010 (.025 mg%) by 40 weeks of gestational life (42–46).

As the muscle mass of the fetus increases and glomerular function matures, the creatinine concentration of fetal urine increases and is reflected by higher concentrations in amniotic fluid. Beyond 36 weeks, concentrations in excess of 2 mg% are normally found (38, 47–50). In mothers with chronic renal disease, toxemia, and diabetes mellitus, the amniotic fluid creatinine is elevated over that of maternal serum (49). However, the feto-maternal gradient remains appropriate for the gestational age. One must know the maternal serum creatinine before proper interpretation of the absolute concentration of creatinine found in amniotic fluid can be made. Cases of intrauterine growth retardation as well as renal agenesis have been reported where the creatinine concentration in amniotic fluid was lower than expected for the gestational age (51, 52).

*Amniotic fluid lecithin.*—The primary objective of estimating fetal age is the prevention of neonatal respiratory distress. The respiratory difficulties of these infants are attributable to a deficiency of alveolar phospholipids, especially lecithin, which results in high alveolar surface tensions, instability, and atelectasis (53). The alveolar lecithin normally present in mature fetuses is produced by special alveolar cells (54) and extruded into the alveolar fluid, which eventually is excreted into the amniotic space. The synthesis of lecithin by fetal alveolar cells begins as early as the 22–24th week of pregnancy, but increases markedly, according to Gluck et al (55), at about the 37th week of pregnancy. This sudden maturing of fetal lung function and increase in alveolar lecithin production has made 37 weeks of pregnancy consonant with near immunity from newborn respiratory problems. That a number of other fetal events (exfoliation of fat-staining cells, increased creatine excretion) also occur at about 36–37 weeks is important because these can be used as indirect indications of fetal lung maturity.

Graven (56) theorized that amniotic fluid concentrations of lecithin might be used to predict those babies that would have the respiratory distress syndrome. Since that time a number of investigators (57–61) have demonstrated the value of amniotic fluid lecithin concentration in determining fetal maturity. Variations due to changes in amniotic fluid volume rather than alterations in pulmonary secretion of lecithin appear to be differentiated by use of the lecithin to sphingomyelin ratio, according to Gluck (62). Prior to the 36th week the fetal lung produces lecithin and sphingomyelin in nearly equal quantities (ratio of 1:1). However, the increase in lecithin production at 36 weeks is not accompanied by a similar increase in sphingomyelin production, so that the ratio rises to 2:1 or greater. There is current controversy as to the optimal methodology for assaying amniotic fluid lecithin (58, 63, 64), and not all investigators have found that low lecithin concentrations or low lecithin-sphingomyelin ratios are inevitably associated with the respiratory distress syndrome (58, 64, 65). However, there is little disagreement that these measurements are useful as an index of fetal lung maturity.

The disadvantage of this analysis, the requirement of special laboratory techniques and facilities, has been reduced by the recently described "shake test" (60). This test capitalizes on the "foaming" characteristics of lecithin in ethyl alcohol. The amniotic fluid is simply agitated in varying dilutions with 95% ethanol. The observance of foam at 1:2 dilution indicates ample lecithin concentration. The absence of foam at 1:1 dilution indicates insufficient lecithin concentration. The test is simple, although the technique must be followed carefully.

### ULTRASOUND

The fetus and placenta in utero are particularly suited to examination by ultrasound, and the method holds exciting promise as a major advance in fetal diagnosis. In the assessment of fetal maturity, it is uniquely applicable early in pregnancy, a distinct advantage over those studies of amniotic fluid described above. The latter are of value in determining when the pregnancy has exceeded 36 weeks, but not in dating the pregnancy prior to that time. Ultrasound is most accurate prior to the 30th week of pregnancy (7, 66), so the two techniques together provide the obstetrician with the means for assessing fetal maturity throughout the major portion of pregnancy. In addition, ultrasound is helpful in preventing complications associated with amniocentesis. The location of the placenta can be determined beforehand, so that placental laceration is avoided.

The ultrasound generally used is emitted by a transducer at a frequency of 2.0 Mc/sec at a rate of $\sim$400 impulses/sec. Sound waves are reflected at interfaces between tissues of differing physical properties and these echoes are detected by the transducer and displayed on an oscilloscope screen (67, 68).

Ultrasonic examination may reveal the presence of the intrauterine pregnancy "ring" as early as the 6th to 8th week, and the fetal head is clearly identified by the 14th week (68). A number of investigators have found an excellent correlation between the biparietal diameter measured by ultrasound and length of gestation (69–72). The transducer is posititioned until a midline intracranial echo (probably falx cerebri) is depicted on the oscilloscope screen, thus assuring that the measuring beam is traversing the fetal head at right angles (Figure 1). Campbell compared his ultrasound biparietal measurements with caliper measurements obtained after delivery and reported a difference of less than 2 mm in 94% of cases and a difference of less than 1 mm in 66% (66).

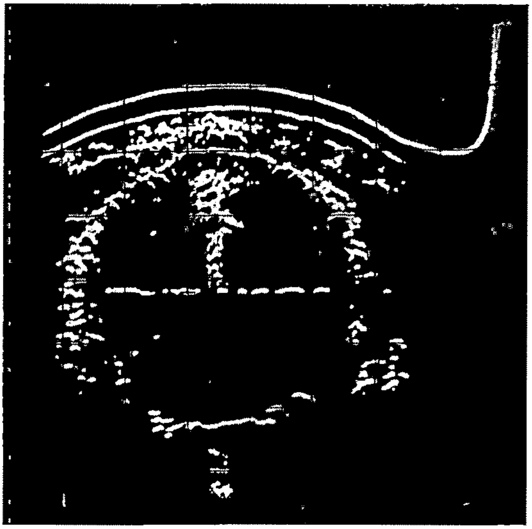

FIGURE 1. Ultrasonogram of fetal head at 35 weeks. The midline echo assures that the biparietal measurement is taken perpendicular to the falx cerebri.

The mean biparietal diameter and standard deviations for known lengths of gestation have been determined for several patient populations (Figure 2). Measurements obtained between 20 and 30 weeks are the most reliable (66). Sequential studies done over several weeks improve the accuracy of estimation of the duration of pregnancy (71, 72). In the event of retarded intrauterine growth, the fetal brain is one of the last organs to exhibit a significant reduction in size, so that the biparietal diameter usually remains appropriate for gestational age (73, 74). If the biparietal diameter is smaller than anticipated, prematurity can be differentiated from severe growth retardation on the basis of the incremental change observed over a one to two week time span (70). The rapid increase normally observed in early pregnancy would not be observed in the case of severe growth retardation.

One of the major advantages of ultrasonic examination is its apparent safety

(75). In a careful review of 1114 patients subjected to ultrasonic examination, Hellman et al (67) were unable to detect any increased incidences of anomalies in the infants at birth. Abdulla et al (76) examined the white cells in cell cultures from mothers and fetuses exposed to ultrasound and could demonstrate no increase in the number of chromosomal aberrations.

The older radiologic techniques of determining skeletal maturation of the fetus are only useful after 36 weeks and are excessively variable. Because of the radiation hazards to the fetus (77), their continued use should be discouraged.

## POSTNATAL ESTIMATION OF GESTATIONAL AGE

The validity of intrauterine measurements of gestational age requires correlation with the assessment of gestational age of the delivered infant. Techniques for the latter have been developed to far greater precision in the past decade, since the recognition that birthweight does not necessarily correlate well with gestational age.

Infants are now categorized into small (SGA), appropriate (AGA), and large for gestational age (LGA). As noted in reports from a number of centers, this

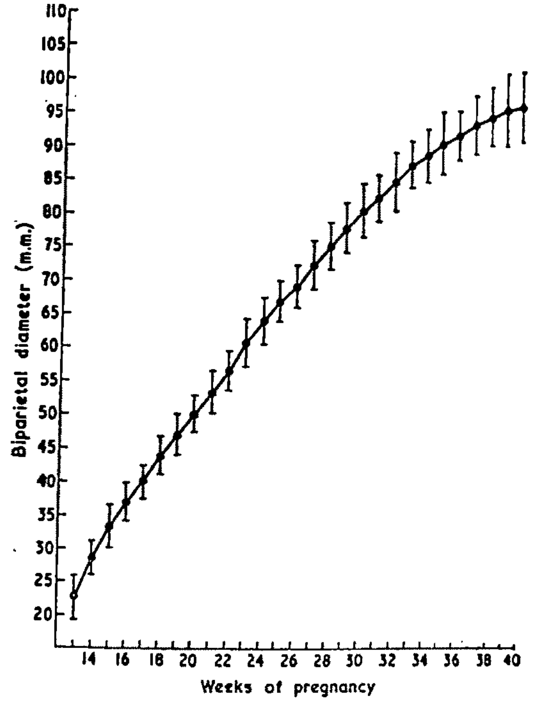

FIGURE 2. Mean biparietal diameters (mm) ± 2 SD for each week of pregnancy from 13 weeks to term (1029 measurements). From (70); reproduced by permission.

permits far better anticipatory care of the newborn and is associated with different morbidities and mortalities (73, 78–80).

The gestational age of the delivered infant is derived from the sum of the numerical score assigned to each of the following parameters: anthropometric measurements, external physical characteristics, neurologic behavior, and motor conduction velocity (81–85). All but the conduction velocity measurements are easily performed in the nursery. For the AGA and LGA infants the 95% confidence limits are ±1 week of gestational age, and it is ±2 weeks for SGA.

## SUMMARY

Limitations in precisely estimating gestational age are the variation between the LMP and conception, degree of reproducibility of the methods, and biologic variation in maturity at a given gestational age. The combination of the aforementioned criteria for judging fetal maturity with the independently derived measurements on the delivered infant now permit much greater quality in the perinatal management of current and future populations.

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# TRANSFER FACTOR THERAPY IN IMMUNE DEFICIENCY STATES[1]

7070

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## TRANSFER FACTOR

### INTRODUCTION

Transfer factor is a dialyzable extract of immune leukocytes capable of transferring cellular immunity from a skin-test positive donor to a skin-test negative recipient. It was discovered by Lawrence in 1955 (1). While working on the passive transfer of cellular immunity by intact leukocytes, he noted that passive transfer could also be induced using nonviable lymphocytes. Pursuing this phenomenon further, he found that leukocyte homogenates could transfer cellular immunity. Lawrence characterized the active factor in the leukocyte homogenate and discovered that activity was lost after dialysis. He then examined the diffusate (that material which bathes the dialysis bag) for the lost activity. After concentrating the diffusate he not only found that he could passively transfer the skin-test reactivity concomitant with the donor specificities, but also that the activity of the extract was intensified.

Although transfer factor was first described many years ago, it was not used prophylactically or therapeutically until we reported its use in a patient with the Wiskott-Aldrich syndrome (2). Since that time, immunologists universally have become interested in the therapeutic benefits of transfer factor, and it is currently being used in a wide variety of disorders associated with defects in cellular immunity.

### CHEMICAL CHARACTER OF TRANSFER FACTOR

Many attempts to characterize the active moiety in transfer factor have met with only moderate success. To date, the following chemical characteristics of transfer factor are known: It appears to be a molecule less than 10,000 in molecular weight (3), and some investigators have reported it to be in the range of 700–4000 (4). It is resistant to DNase treatment, bovine pancreatic RNase treatment, and has been reported by Lawrence to be heat labile (5). Its heat stability is presently under further investigation in our laboratory. The activity of transfer factor probably resides in either an oligonucleotide or a small polypeptide, both of which are present in crude preparations. The oligonucleotide is reported by some investigators to contain adenine, guanine, and cytosine with a ribose phosphate backbone (6).

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Because a number of different polypeptides are present in the transfer factor preparation, sequence analysis is presently impossible. One important point regarding the peptides is the low concentration of tyrosines and phenylalanines (those amino acids that absorb energy at 280 m$\mu$) which are often used to measure concentrations of proteins spectrophotometrically (7). Whether the peptide and nucleotide are bound together in the native transfer factor preparation is unknown.

A major difficulty in characterizing the active moiety of transfer factor is that until recently no animal models had been successful (Bloom & Chase 8). Burger & Jeter (9) recently reported success in transferring sensitivity to dinitrochlorobenzene (DNCB—a contact sensitivity-producing agent believed to represent delayed hypersensitivity) between rabbits using dialyzable extracts. They found that the extract had an oligonucleotide and a small polypeptide similar to human transfer factor. No antigen was noted in the active preparation although many techniques were used. They suggest that the success of transfer was due to the rapid processing of lymphocytes following their removal from the lymph nodes of the experimental animals. They claim that transfer factor activity is extremely labile in animals and is often lost during processing; a markedly high donor recipient ratio (usually 8:1) was generally required. The authors, using the same technique, could not transfer purified protein derivative (PPD) sensitivity. These results, although exciting, leave a number of questions unanswered about transfer factor in animals.

Another major difficulty in characterizing the active moiety of transfer factor has been the distinct lack of reliable in vitro systems. Fireman et al (4) have reported transfer factor activity in vitro. Leukocytes derived from a donor devoid of skin reactivity to PPD were incubated in the presence of PPD with transfer factor from a tuberculin-positive donor. The appropriate controls were included. Following in vitro sensitization, an increase in the number of morphologically transformed cells (blast cells) was noted. To our knowledge, these investigators have not repeated these studies nor have they modified the technique by using radioactive incorporation or other quantitative techniques.

Lawrence & Valentine (10) have studied passive transfer of transfer factor activity in vitro. The technique of radioactive thymidine incorporation has yielded preliminary observations indicating that "a small, but consistent, number of lymphocytes will transform and divide when cultured in the presence of tuberculin transfer factor and PPD." They further report that supernatants of cultures of sensitized lymphocytes with antigen can cause an increased incorporation of radioactive thymidine in cultures of nonsensitive cells; this action increases when additional antigen is added to the secondary culture. Their active agent is nondialyzable and therefore not consistent with dialyzable transfer factor. Many lymphocyte factors may be causing this laboratory phenomenon.

Several other reports have demonstrated in vitro transfer of immunity by nondialyzable subcellular extracts. Whole cell lysates from sensitized lymphocytes can transfer (to nonsensitized lymphocytes) the ability to undergo increased radioactive thymidine incorporation or to produce migration inhibitory factor (MIF) in response to antigen stimulation (11–14). Although many investigators have called these active preparations "transfer factor," it does not appear to be the same substance that we are using in our human studies.

## SPECIFICITY OF TRANSFER FACTOR

Our carefully controlled studies (2, 15) and those of other investigators (16) consistently show that transfer factor confers the skin-test specificity of the donor and appears to have no nonspecific "booster effects." Lawrence (3) demonstrated that when lymphocytes cultured from a donor showing sensitivity to more than one antigen are stimulated with one of the specific antigens, the cells will secrete into the supernatant only those transfer factors related to the specific challenging antigen. That is, when challenged in culture with PPD, lymphocytes from a donor who is skin-test sensitive to PPD and diphtheria will secrete only PPD-positive transfer factor into the supernatant. The supernatant will be unable to confer diphtheria skin-test reactivity. The cell button, however, will confer diptheria sensitivity but will be devoid of PPD activity when processed (17). This indicates that transfer factor is specifically released for specific antigens, or possibly for families of antigens, and that it is preformed and stored in lymphocytes. Antigen challenge may also induce further synthesis of transfer factor.

## MODE OF ACTION OF TRANSFER FACTOR AND ITS ROLE IN THE NORMAL CELLULAR IMMUNE RESPONSE

The mode of action of transfer factor is presently unknown. Our group feels it probably induces the synthesis of a specific receptor site on a previously uncommitted lymphocyte. To illustrate our reasoning for this assumption, we must first discuss the cellular immune response in greater detail, covering the biochemical events and currently available methods of assessment.

According to the latest theories, the cellular immune reaction in a previously sensitized host is initiated when an antigen strikes a specific receptor site on a sensitized T-lymphocyte. The character of the antigen when it strikes the lymphocyte is unclear. Possibly it is first processed in a macrophage.

A complex antigen is first processed by the macrophage: this may be a mycobacterium, a tumor cell, or a whole virus. The processed antigen is presented to a receptor site on the sensitized lymphocyte. The nature of the specific receptor on the T-lymphocyte has not been completely defined. Some think that it is a specific immunoglobulin, chemically similar to circulating IgG; others think it is a non-immunoglobulin protein. Whatever the chemical nature of this receptor may be, it is rapidly turning over in a dynamic cell membrane.

**TABLE 1. Factors secreted by activated lymphocytes in the cellular immune response**

A. Factors acting primarily on other lymphocytes
  1. transfer factor
  2. mitogenic factor
B. Factors acting primarily on macrophages
  1. migration inhibitory factor (MIF)
  2. macrophage activation factor
  3. macrophage aggregation factor (MAF)
  4. chemotactic factor
  5. antigen-dependent migration inhibitory factor
C. Necrotizing factor
  1. lymphotoxin
D. Antiviral and other factors
  1. interferon
  2. skin-reactive factors
  3. immunoglobulins

The lymphocyte then undergoes many complex reactive steps, resulting clinically (and in the laboratory) in cellular immunity. Table 1 shows the factors secreted by activated lymphocytes in the cellular immune response.

*Factors acting primarily on other lymphocytes.*—The sensitized lymphocyte secretes transfer factor which, as stated above, is preformed and stored in the lymphocyte cytoplasm or on the membrane. New specific transfer factor may also be synthesized at this time. The biological role of transfer factor appears to be to increase the number of specifically sensitized lymphocytes by recruitment of previously uncommitted cells.

It is unknown whether mitogenic factor is preformed in the lymphocyte or synthesized at the time of antigen challenge. It is apparently a glycoprotein agent enabling lymphocytes to undergo blastogenesis and divide into daughter cells (18–20). The biological advantage of both transfer factor and mitogenic factor is that they can increase the number of specifically sensitized lymphocytes not only in a local area of antigen challenge but indeed throughout the body.

*Factors acting primarily on macrophages.*—While secretion of other mediators of cellular immunity is antigen initiated, the action of these mediators appears to be primarily nonantigen specific:

Migration inhibitory factor (MIF) is believed to be a glycoprotein which migrates electrophoretically in the prealbumin range and has a molecular weight of 35,000–50,000. It appears to be synthesized at the time of antigen challenge since MIF secretion in vitro can be inhibited by inhibiting protein synthesis (21). MIF apparently functions biologically by preventing "wandering macrophages" from passing the site of infection.

Macrophage activation factor, with a molecular weight ranging from 25,000–80,000, increases macrophage ameboid activity, glass adherence, phagocytosis, and death of intracellular organisms (22–24).

Macrophage aggregation factor (MAF) may be identical to MIF and seems to have the same biological function (25, 26).

Chemotactic factor is a protein with a molecular weight of ~60,000 which attracts macrophages toward a concentration gradient. It differs chemically from eosinophil chemotactic factor (secreted by stimulated mast cells and platelets), and the polymorphonuclear neutrophilic chemotactic factors, one of which is secreted by lymphocytes while the others are products of activation of C3 and C5 (27, 28).

Antigen-dependent MIF acts with specific antigen to inhibit the migration of macrophages (29–31).

The probable biological role of the above-mentioned mediators is to increase the numbers of macrophages locally and activate them to attack and kill the invading antigen.

*Necrotizing factor.*—Lymphotoxin is a protein with a molecular weight of 80,000 which is secreted by activated, sensitized lymphocytes. It appears to destroy numerous cell types from many species including mouse L-cells, continuous human lymphocyte cell lines, fibroblasts, macrophages, and red blood cells. Different cell types show varying degrees of sensitivity with lymphocytes being relatively resistant, which thus avoids destruction of the effector cell in the reaction (32–34).

All of the previously mentioned factors appear to act in concert to create the classical lesion of delayed hypersensitivity—the positive skin test; granulomas (localized areas of lymphocytes and macrophages surrounding a necrotic center) may also be produced by these mediators.

*Antiviral and other factors.*—Other agents secreted by activated lymphocytes include interferon (35, 36), immunoglobulins (37), and skin-reactive factors (38). Interferon is a glycoprotein with a molecular weight of approximately 25,000 which inhibits viral replication in the infected cell. The immunoglobulins are poorly characterized at present but could be specific cytotoxic antibody and/or antiviral agents. Skin-reactive factors are nondialyzable moieties (possibly proteins) which cause induration, erythema, and mononuclear infiltrates in the skin-test site. They may be combinations of all of the previously mentioned factors.

## Assessment of Cellular Immunity

Table 2 outlines some of the present methods of assessing cellular immunity. This may be done both in vivo and in vitro. The best and most accurate tests for cellular immunity in vivo are the skin tests. These are important tools in evaluating patients for possible defects in cellular immunity; the observation of a positive skin test indicates the integrity of the afferent, central, and efferent limbs of

TABLE 2. Presently available methods of assessment of cellular immunity

- I. IN VIVO
  - A. skin tests
  - B. skin-graft survival
- II. IN VITRO
  - A. Lymphocyte transformation
    - 1. nonspecific (plant mitogens)
    - 2. specific (specific antigens)
  - B. Macrophage migration inhibition factor
    - 1. specific antigens only
  - C. Rosette-forming cells
    - 1. nonspecific

the cellular immune system. The keys to success in their use are: 1. The use of the complete battery of six skin-test antigens, 2. repetition of the tests in higher antigen concentration when the tests are negative with intermediate strength, and 3. careful observation and recording of the results in millimeters of erythema and induration at 24 and 48 hr. This will aid future observers much more than a simple recording of positive or negative. Table 3 outlines the antigens used by our group in the routine battery of skin tests.

TABLE 3. Antigens

| Antigen | Trade name | Strength | | Source |
|---|---|---|---|---|
| | | Intermediate | Second | |
| Candida | Dermatophytin 0 | 1:100 | 1:10 | Hollister-Stier Labs, Spokane, Washington |
| Coccidioides | Coccidioidin | 1:100 | 1:10 | Cutter Labs, Berkeley, California |
| Mumps | Mumps | — | — | Eli Lilly & Co., Indianapolis, Indiana |
| PPD | PPD (stabilized solution) | 2 μg/cc | 50 μg/cc | Connaught Labs, Toronto, Canada |
| Streptokinase-Streptodornase | Varidase | 40 *u*/10 *u* | 400 *u*/100 *u* | Lederle Labs, American Cyanamid Co., Pearl River, N.Y. |
| Trichophytin | Dermatophytin | 1:30 | — | Hollister-Stier Labs, Spokane, Washington |

There are several in vitro assays of cellular immunity, including the following:

1. Lymphocyte transformation from nonspecific mitogenic factors and specific antigens can be measured in various ways. Nonspecific mitogens include phytohemagglutinin (an extract of red kidney beans), pokeweed mitogen (an extract of pokeweed), and concanavalin A (an extract of the jack bean). They cause lymphocytes to undergo mitogenic changes and divide into daughter cells. The effect of these mitogens can be assessed either by counting the number of blasts in an aliquot of the lymphocyte culture or by quantifying the uptake of tritium-radiolabeled nucleotide precursors, usually thymidine, into newly formed DNA. Specific antigens will also cause sensitive lymphocytes to respond in culture by undergoing mitogenic changes and dividing into daughter cells. The number of cells responding to specific antigens is smaller than that responding to nonspecific mitogenic factors. However, the magnitude of response can be measured in the same manner as that of the nonspecific mitogens. The technique has been described in detail in many reports (18–20, 39) and is presently accepted as a manner of assessing lymphocyte transformation. Figure 1 shows a schematic representation of this technique.

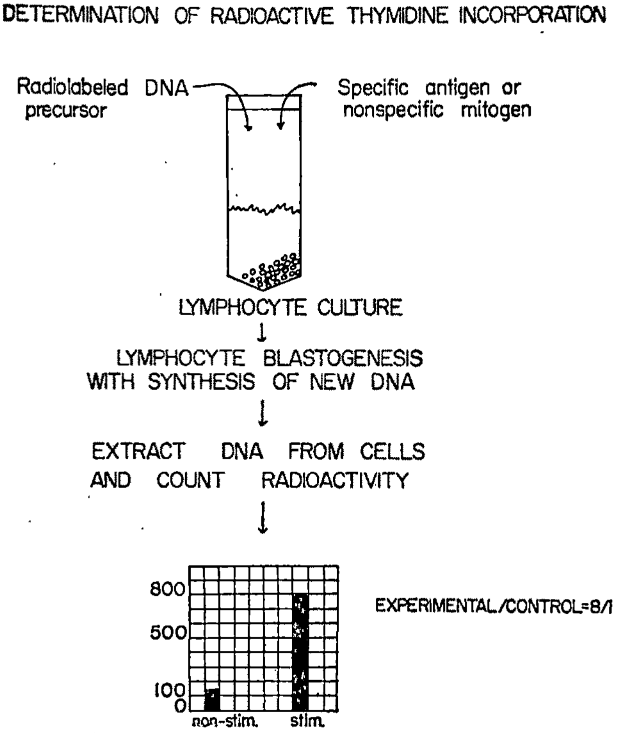
DETERMINATION OF RADIOACTIVE THYMIDINE INCORPORATION
Radiolabeled DNA precursor
Specific antigen or nonspecific mitogen
LYMPHOCYTE CULTURE
LYMPHOCYTE BLASTOGENESIS WITH SYNTHESIS OF NEW DNA
EXTRACT DNA FROM CELLS AND COUNT RADIOACTIVITY
800
500
100
0
non-stim.
stim.
EXPERIMENTAL/CONTROL=8/1

FIGURE 1. Schematic representation of assay for lymphocyte transformation.

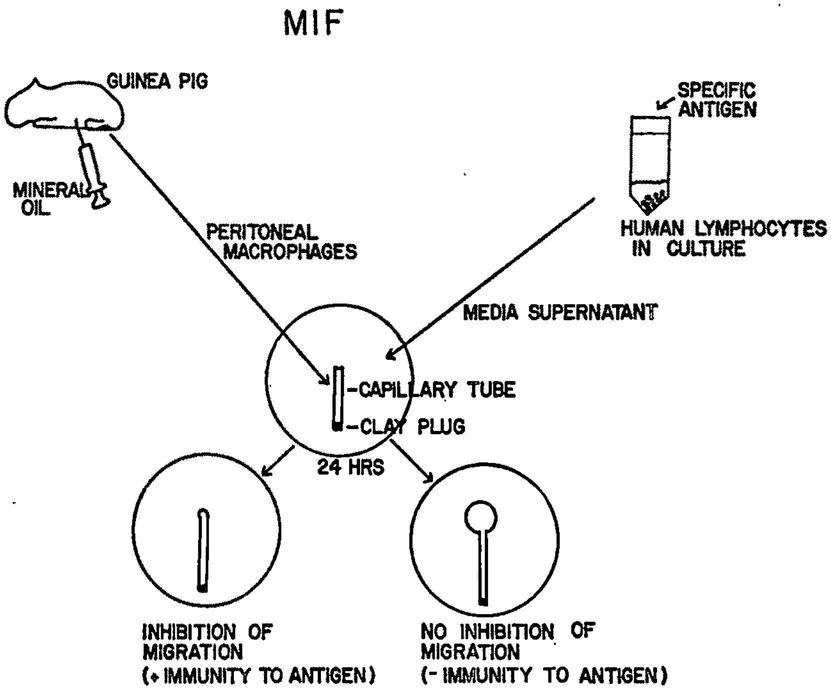
MIF
GUINEA PIG
MINERAL OIL
PERITONEAL MACROPHAGES
SPECIFIC ANTIGEN
HUMAN LYMPHOCYTES IN CULTURE
MEDIA SUPERNATANT
-CAPILLARY TUBE
-CLAY PLUG
24 HRS
INHIBITION OF MIGRATION (+ IMMUNITY TO ANTIGEN)
NO INHIBITION OF MIGRATION (- IMMUNITY TO ANTIGEN)

FIGURE 2. Schematic representation of assay for Macrophage Migration Inhibition Assay (MIF).

2. Upon challenge by an antigen to which the cells are sensitive, cultures of peripheral lymphocytes will elaborate and secrete into the supernatant macrophage inhibitory factor (MIF). The presence or absence of this factor in the supernatants of challenged lymphocyte cultures can be assessed by the ability of that supernatant to inhibit the migration of guinea pig peritoneal macrophages from a capillary tube, the so-called indirect technique. The technique is schematically represented in Figure 2. Figure 3 shows a typical migration inhibition pattern.

3. A percentage of peripheral lymphocytes (rosette-forming cells—RFC) will form rosettes when incubated with sheep red blood cells (40). Much evidence convincingly suggests that thymic-derived lymphocytes (T-cells) are responsible for this. The mechanism of this reaction is unclear but may be related to the large number of cross-reacting antigens on the sheep red cell membrane which may bind to various cross-reacting specific receptor sites on sensitive T-lymphocytes. These rosette-forming cells are also capable of undergoing blast transformation when stimulated with phytohemagglutinin (41), further substantiating that they are T-lymphocytes. The number of circulating RFC in normal individuals ranges

from 17–25% of the total circulating lymphocytes (this test measures only a certain subpopulation of T-cells; the total number of circulating T-cells is greater).

Patients with untreated congenital immune deficiences have very low numbers of circulating rosette-forming cells (42), as do patients with progressive malignant disease (43). When treated with transfer factor, patients with the Wiskott-Aldrich syndrome often respond dramatically. This response is marked by the induction of positive skin-test reactivity, MIF production, disappearance of eczema (when present), and marked reduction in the frequency of infections. Those who demonstrate the clinical and in vitro response to transfer factor also consistently show a significant rise in the number of rosette-forming cells in the peripheral blood (44), which suggests that transfer factor induces the synthesis of specific receptor sites on previously uncommitted lymphocytes. These lymphocytes then engage in cellular immune reactions in vivo.

In many of the patients we have treated with transfer factor, we have been able to demonstrate a significant dichotomy between the increased DNA synthesis caused by antigen or mitogen on one hand, and production of migration inhibitory factor, skin-test reactivity, and rosette-forming cell rise on the other. We have consistently found that patients incapable of showing an increased DNA synthesis in response to nonspecific mitogens and antigens prior to transfer factor

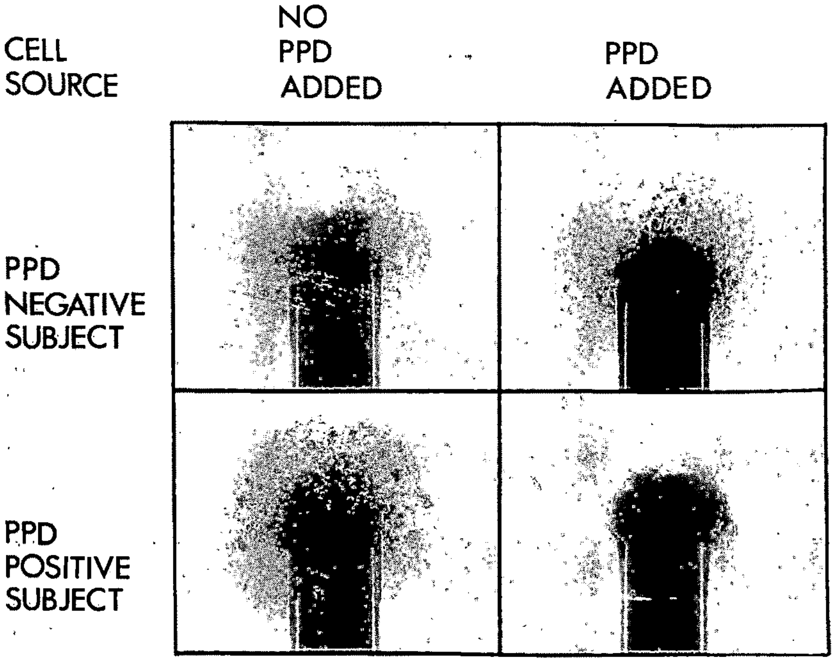

FIGURE 3. Typical MIF patterns.

therapy continue to manifest this defect after therapy despite the fact that their lymphocytes acquire the ability to produce MIF and positive skin tests to specific antigens and rosette-forming cells. This suggests that those cells responsible for the increased DNA synthesis are either in a different growth-cycle phase or from an entirely different population of lymphocytes than those responsible for MIF production and skin-test reactivity. Normal subjects treated with transfer factor or subjects with malignant diseases who previously had normal cellular immunity are often rendered capable of mounting blastogenic responses, along with MIF, positive skin tests, and rosette-forming cells. This implies that blastogenesis can be induced as a secondary effect of transfer factor therapy in those patients genetically capable of mounting such a response.

## IMMUNE DEFICIENCY STATES: CLASSIFICATION, DESCRIPTION, CLINICAL AND LABORATORY RESPONSE TO TRANSFER FACTOR THERAPY

### INTRODUCTION

Immune deficiency states can be either congenital or "acquired." Congenital immune deficiency states were first recognized as clinical entities after the onset of the antibiotic era. Prior to the liberal use of antibiotics, the majority of patients with immune deficiency states died of infectious diseases before diagnosis. Acquired immune deficiency states have become prominent clinical entities since the increased knowledge of cellular and antibody immune deficiencies has become available. Added impetus has been given to this field by the onset of transplant surgery and the use of immunosuppressive therapy in malignant diseases.

In this section we will describe the currently held scheme for the ontogenesis of the immune response, attempt to classify and describe congenital and acquired immune deficiency states, and roughly place these states on the scheme of the ontogenesis of the normal immune response. We will then describe the observed or expected laboratory and clinical response to transfer factor therapy in each disease.

### DEVELOPMENT OF THE IMMUNE RESPONSE

Figure 4 shows a schematic representation of a hypothesis of the development of the immune system in humans. A primordial stem cell, the reticular anlage cell, located in the embryonic bone marrow or bone marrow precursor, gives rise to a series of stem cells which mature into different components of the reticular system. As shown, the stem cell matures into the precursors of the erythropoietic series, the thrombopoietic series, macrophages, the myeloid series, and the lymphoid series of cell lines. The lymphoid stem cell can migrate in one of two directions. It can pass through the thymus where it is acted upon by some humoral factor from the epithelial component of the thymus (a derivative of the third and fourth embryonic pharyngeal pouches). After traversing the thymus, these small mature lymphocytes then pass to the peripheral lymphoid system where they populate the pericortical and deep cortical centers of the lymph nodes and the white pulp of the spleen. From there, these cells may involve themselves in cell-mediated immunity.

The lymphoblast may also migrate to some as yet undetermined region, analogous to the bursa of Fabricius in chickens, a lymphoid organ located over the cloaca which appears to have activity in the development of the humoral im-

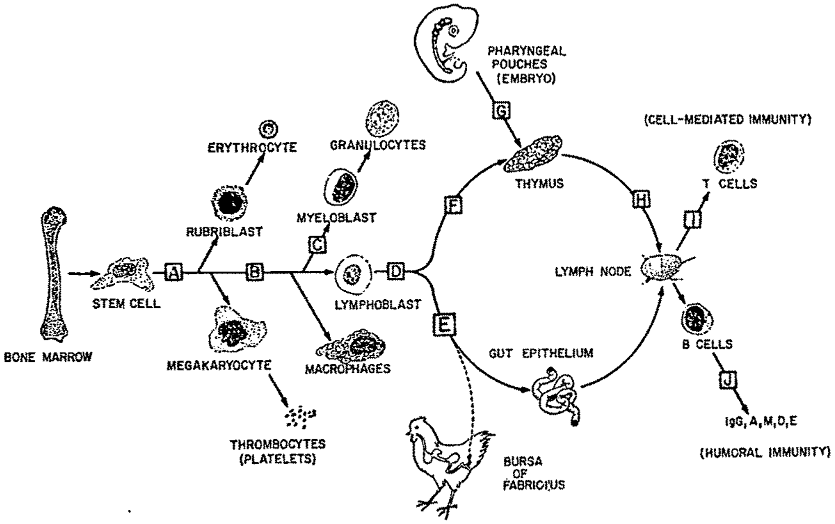

Figure 4. Development of the immune response in humans. From: Levin, A. S., Spitler, L. E., Fudenberg, H. H. *Immune Deficiency States, An Introduction to Clinical Allergy*, ed. B. F. Feingold. Springfield, Ill.: Charles C Thomas

The site of the postulated developmental defect in each disease is indicated by a letter.

*A.* Not reported—probably a lethal defect
*B.* Reticular agenesis
*C.* Granulocyte defects
  1. Chronic granulomatous disease
  2. Chediak Higashi
  3. Job's syndrome
*D.* Combined dual system deficiencies
  1. X-linked recessive
  2. Autosomal recessive
*E.*[a] B-cell defects
  1. X-linked infantile hypogammaglobulinemia
*F.* T-cell defects
  1. Wiskott Aldrich
  2. Ataxia telangiectasia
  3. Nezeloff
*G.* Thymic epithelial arrests
  1. DiGeorge
  2. Nezeloff
*H.* Acquired cellular immune defects
  1. Infectious
  2. Malignant
*I.* Malignant replacement of immune competent lymphocytes
*J.* Selective immunoglobulin disorders

[a] Patients with diminished levels of circulating immunoglobulins in all three major classes may have defects in the lymphoblast stem cell before or after it passes through the bursal equivalent or indeed may have a defect in the development of the gut epithelial component.

mune system. From the bursal equivalent these lymphoid cells may migrate to the germinal centers of the lymph nodes, there to become the large immune globulin-secreting lymphoid cells which then take on the character of plasma cells. These cells also migrate back to the bone marrow where as plasma cells they continue to secrete the immune globulins IgG, IgA, IgM, IgD, and IgE.

## CLASSIFICATION OF IMMUNE DEFICIENCY STATES AND RESPONSES TO TRANSFER FACTOR THERAPY

Classifications of immune deficiency states are misleading since we are just beginning to understand humoral and cellular immunity, especially as they relate to human disease. What we observe clinically and in the laboratory as immunologic reactions represents the endpoint of thousands of interdependent biochemical and developmental steps. A defect in one or more of these steps may manifest itself clinically as an immune deficiency state. Thus two patients manifesting the identical clinical syndrome and, indeed, identical laboratory findings may not have identical immunologic defects. Any classification of immune deficiency disorders is therefore weakened by the fact that we do not know the basic biochemical defects involved in these diseases.

*Congenital or hereditary disorders.*—Despite our uncertainties, we will attempt to roughly classify these diseases as those involving defective humoral (B-cell system) and intact cellular immunity (T-cell system), those involving defective cellular (T) and intact humoral immunity (B), mixed deficiencies (B and T), variable deficiencies, and neutrophil dysfunction syndromes (Table 4). These categories represent quantitative differences between the two major immune systems; however, none of the authors has ever seen a patient with an absolute absence of either system. The site of the postulated defect in each disease state is indicated by a letter in Figure 4.

*Defective humoral (B-cell system), intact cellular (T-cell system)*: These include the specific disorders outlined below.

1. Infantile X-linked agammaglobulinemia—"Bruton" (Letter *E* on Figure 4). Infantile X-linked agammaglobulinemia patients were first reported by Bruton in 1952 (45). This is a rare disease characterized clinically by marked susceptibility to recurrent pyogenic infections. Conjunctivitis, otitis media, pneumonia, cellulitis, and gastrointestinal infections (especially Giardia lamblia) are common in these children. Laboratory findings include markedly diminished levels of all measured serum immunoglobulins. Most of these patients have small amounts of one or more classes of immunoglobulins; however, they cannot be immunized by routine antigens used to induce humoral immune responses. This implies that although they have the primordial machinery to make immunoglobulins, those that are made may be "nonsense immunoglobulins." The cellular immune system in these patients appears to be generally intact (46, 47). Bone marrow aspirations show a marked paucity of mature plasma cells. Lymph node biopsies reveal abundant small mature lymphocytes in pericortical and deep cortical regions and the absence of germinal centers and plasma cells. X rays of

the nasal pharyngeal regions reveal a marked paucity of lymphoid tonsils and adenoid tissue.

Patients with this syndrome, although they appear to have a markedly diminished humoral immune system, often manifest the stigmata of rheumatoid arthritis and atopic allergy. Although the basic pathogenetic mechanism of the arthritis is unclear, it is very possibly a result of cellular immune reactions against tissues. Perhaps these represent the lack of control of T-cells reacting against self-antigens. Alternatively, it could represent activation of a latent virus in the absence of antibody formation against the agent. The atopic reactions in these patients are commonly anaphlactoid and urticarial. These could quite possibly be mediated through some bypass mechanism involving activation of the third

TABLE 4. A. CONGENITAL IMMUNE DEFICIENCY STATES[a]

- A. Defective Humoral "B-Cell System," Intact Cellular "T-Cell System"
  - 1. Infantile X-linked agammaglobulinemia—"Bruton type"
  - 2. Selective immunoglobulin disorders
  - 3. Transient hypogammaglobulinemia of infancy
  - 4. Non-sex-linked primary immunoglobulin aberrations—"adult acquired"
- B. Defective Cellular—Intact Humoral
  - 1. Thymic and parathyroid aplasia—DiGeorge
  - 2. Autosomal recessive thymic aplasia with lymphopenia—"Nezeloff"
- C. Mixed Deficiencies
  - 1. Ataxia telangiectasia
  - 2. X-linked dual system deficiency—thymic alymphoplasia
  - 3. Autosomal recessive dual system deficiency—Swiss type
- D. Variable Deficiencies
  - 1. Mucocutaneous candidiasis
  - 2. Wiskott-Aldrich syndrome
- E. Granulocyte Defects
  - 1. Chronic granulomatous disease
  - 2. Job's syndrome
  - 3. Chediak-Higashi syndrome

[a] The theoretical location of the defect causing each deficiency state is represented by the capital letters in Figure 4.

B. ACQUIRED IMMUNE DEFICIENCY STATES

| | | |
|---|---|---|
| Infectious: | Sarcoid | |
| | Tuberculosis | Intact humoral |
| | Coccidioidomycosis | Variable cellular defects |
| Malignant: | Lymphoreticular | |
| | Carcinoma | Variable defects |
| | Sarcoma | |
| Iatrogenic: | Immunosuppressive therapy | Variable defects |

component of complement (C3) via plasma kinin systems in the absence of antigen-antibody complexes (48).

The basic biochemical defect in this disease appears to be the maturation of the normal B-cell system prior to the cells' passage through the bursal equivalent and/or in the maturation of that bursal equivalent itself.

Since cellular immunity appears to be reasonably intact in these patients and transfer factor affects only cellular immunity, it seems unlikely that this mode of therapy would be helpful. On selective occasions, however, such as when the patient is overwhelmed with bacterial infection and apparently unable to handle it with plasma infusions and antibiotics, transfer factor may enhance his cellular immune competence and thereby allow him to recover.

2. Selective immunoglobulin disorders (Selective IgA deficiency—letter *J* on Figure 4). The most common selective immunoglobulin disorder occurs when IgA is markedly diminished or absent while IgG, IgM, IgD, and IgE are normal (49). These patients were previously thought to be asymptomatic; however, a high incidence of upper respiratory infections, asthma, gastrointestinal disorders, and autoimmune disorders have been observed (50). Secretory IgA is also diminished in these patients. A high frequency of antiruminant antibodies are also seen, possibly as a result of the lack of secretory IgA. Cellular immunity appears to be intact (51).

The defect in these patients is polymorphic. Some appear to have structural gene defects as no IgA can be found in their cells. Others appear to have regulatory defects such as diminished synthesis or secretion (52–55). The ultimate result of these defects is the marked absence of circulating and secretory IgA.

3. Selective immunoglobulin deficiency. Other selective immunoglobulin disorders involving diminished IgG levels with elevated IgM and at times elevated IgA levels have been reported. Selective IgM deficiencies have also been reported (56). These patients suffer from recurrent pyogenic infections.

4. Specific immune paralysis with normal immunoglobulin levels. A new and possibly common selective immunoglobulin disorder involving patients with recurrent infections having "normal" levels of immunoglobulins is now being recognized at several medical centers (57, 58). When investigated further, these patients appear to have specific immune paralysis or an inability to respond to certain antigenic stimuli, rendering them immune deficient to one or more pathogenic moieties. An example is the patient who apparently received a full complement of DPT vaccinations but still demonstrated a nonimmune Schick response, indicating a specific failure of antibody response to diphtheria antigen.

Generally patients with selective immunoglobulin disorders will have normal cellular immune competence as measured by laboratory tests. However, we have treated one patient with a selective immunoglobulin deficiency (15) who appeared to have depressed cellular immunity in association with debilitation and immunoglobulin disorder; transfer factor enhanced cellular immune competence and appeared to accelerate recovery from chronic salmonella diarrhea.

5. Transient hypogammaglobulinemia of infancy (letter *E* on Figure 4). The normal newborn infant is endowed with adult levels of IgG from placental trans-

port of maternal immunoglobulins that disappear from the infant's circulation by four months of age. Endogenous synthesis of IgG begins in the third trimester of pregnancy; levels gradually increase until the infant is three months old when his own IgG becomes the dominant IgG protein in the circulation. The levels then rise gradually until the early teens when they plateau at the adult values. IgM synthesis begins very late in the third trimester of pregnancy and detectable levels are often noted in cord blood. Circulating IgA is often undetectable in the normal newborn cord blood but appears shortly after birth and rises gradually until the teenage period.

In transient hypogammaglobulinemia of infancy the onset of endogenous synthesis of these immunoglobulins is delayed. Such children rarely have problems before three months of age and are often asymptomatic. Probably a majority of infants with this disorder escape detection since they never arouse the suspicion of the clinician; they generally come to the pediatrician at between four and six months of age with recurrent pyogenic infections, mostly minor in nature. A routine immunoelectrophoresis usually reveals the diagnosis. Therapy of these patients is controversial. Some believe that treatment with injections of IgG will reduce the recurrent infections and thereby tide the child over until endogenous synthesis begins. Others feel that exogenous IgG should not be used since it may retard endogenous synthesis of IgG. The decision to treat with injections of gammaglobulin of course depends upon the severity of the recurrent infections and the clinical judgment of the practicing physician. These children generally recover at approximately one year of age, and appear normal after that; any child with an IgG level of less that 200 mg% after one year of age should be suspected of having a permanent hypogammaglobulinemia. The basic defect in this disease may be a result of isoimmunization during pregnancy (59), that is, maternal antibodies directed against genetic markers of the infant's own immunoglobulin (those inherited from the father) may destroy or retard the maturation of plasma cells which would eventually secrete these immunoglobulins.

Transient hypogammaglobulinemia of infancy should *not* be treated with transfer factor. This disease is self-limiting and generally of very low morbidity, and involves primarily defective humoral immunity.

6. Non-sex-linked primary immunoglobulin aberrations—adult "acquired" hypogammaglobulinemia. This disease is generally recognized at the end of the second decade or through the third decade of life, and is characterized by an insidious onset of recurrent pyogenic infections. Upon routine examination, diminished or absent immunoglobulins of all classes are found. The patients apparently had normal immunoglobulin levels during infancy and childhood since they did not develop these recurrent infections at that time. A carrier state can be recognized in relatives (60). The patients often develop lymphoreticular malignancies as the disease progresses.

This disease is primarily an acquired B-cell defect, and these patients clinically appear to have reasonably normal cellular immunity. However, our group (60) has demonstrated a defective blastogenic response to phytohemagglutinin in synchronized cultures of lymphocytes in this disease. The patients suffer from a very high frequency of lymphoreticular malignancies, which implies that cellular immunity is subclinically defective. Transfer factor therapy may therefore be helpful as prophylaxis against spontaneous malignant transformation in the lymphoreticular system.

*Defective cellular (T-cell system) and intact humoral (B-cell system):* The two major disorders of this type are discussed below.

1. Thymic and parathyroid aplasia—"DiGeorge" (letter *G* on Figure 4). During embryogenesis, epithelial components derived from the third and fourth pharyngeal pouches form the upper portion of the pinna of the ear, part of the mandible, part of the aortic arch, the parathyroid glands, and the epithelial component of the thymus gland. Defects in any of these developmental steps will result in deficiencies of the resulting organ system. One such defect involving thymic and parathyroid aplasia and commonly defects in the aortic arch, mandible, and ear lobe has been recognized as the DiGeorge syndrome (61). These patients commonly present with neonatal tetany which cannot be cured by simple calcium infusion and ultimately require parathyroid hormone therapy. After the hypoparathyroidism is treated, the patients will develop recurrent infections and eventually die if untreated. They have normal or elevated serum immunoglobulin levels with lymphopenia, absence of skin-test reactivity, and diminished in vitro parameters of cellular immunity. Some investigators (62) feel that this disease is related to an intrauterine viral infection occurring during gestation. A large number of these patients were recognized in the New England area during the middle of the 1960s; however, very few new patients with this syndrome have been reported in that area, which suggests an epidemic of some mutagenic virus which passed through the population and was dissipated.

DiGeorge patients are athymic because they lack the epithelial components derived from the third and fourth pharyngeal pouches. They have reticular anlage cells which undergo development into the humoral immune system. Development of the cellular immune system appears to lack only that step involving the lymphoid/epithelial interaction within the thymus. Since the appropriate lymphoid precursors are present in the patients, transfer factor might be effective in inducing cellular immunity and clinical benefit.

Patients with the DiGeorge syndrome have been successfully treated with thymic transplants (63, 64). This mode of therapy, however, is extremely hazardous since it has been shown that fetal thymic cells can respond to mitogens and react in mixed leukocyte cultures. Thus they can most probably induce graft-versus-host reactions (65). To date, no reports of DiGeorge patients treated with transfer factor can be found.

2. Autosomal recessive thymic aplasia with lymphopenia—"Nezeloff" (letters *G* and/or *F* on Figure 4). The Nezeloff syndrome appears to be a milder variant of the above-described disorder. These patients appear to have normal parathyroids and aortic arches. They do, however, have thymic aplasia with a markedly diminished T-cell system (66).

The Nezeloff syndrome, like the DiGeorge, may involve the absence of an epithelial component of the thymus and therefore patients with this syndrome may benefit greatly from transfer factor therapy. However, to date there are no reports of Nezeloff patients treated with transfer factor.

*Mixed deficiencies* (letters *D*, *F*, and *J* on Figure 4): These deficiencies include the three disorders described below.

1. Ataxia telangiectasia. Ataxia telangiectasia is an autosomal recessive disease characterized clinically by a progressive familial choreoathetosis, cutaneous telangectasia, and recurrent sinopulmonary infection. These patients have a high incidence of reticuloendothelial neoplasms, and laboratory findings indicate a markedly diminished serum IgA, apparently caused by a decreased synthesis of that immunoglobulin. Some patients have detectable antibodies directed against allogeneic IgA, and some also have markedly diminished IgG levels. They have diminished cellular immunity (T-cell systems) measured by absent skin reactivity to skin-test antigens, and diminished in vitro parameters of cellular immunity (67–71).

Ataxia telangiectasia involves both humoral and cellular immune defects. It appears highly likely that a patient with this syndrome would benefit from transfer factor therapy. To our knowledge, five patients with this syndrome have been treated with transfer factor (72) and clinical improvement was reported in three of these (regression of neurologic symptoms).

2. X-linked dual system deficiency—thymic alymphoplasia (letter *D* on Figure 4). Patients with severe combined dual deficiency disorders generally have diminished immunoglobulin levels in all classes. Some patients with these disorders, however, may have low-normal to normal levels of one class of immunoglobulin. These patients also have severely deficient cellular immune systems with absent skin-test reactivity and diminished in vitro responsiveness of lymphocytes to nonspecific mitogens such as phytohemagglutinin and to specific antigens. They are generally very ill and have problems with pyogenic bacteria, viruses, fungi, and infections from opportunistic organisms. If untreated, these patients rarely survive their early infancy (73–76). This disorder was previously thought to be a defect of the stem cell in the lymphoid line, since patients suffer from severely defective cellular and humoral immunity. As previously stated, however, none of the authors has ever seen a patient who manifests an absolute defect in either immunologic system. With this in mind, our group has attempted transfer factor in a patient with this disorder. The patient, a five month old infant with a family history of X-linked immune deficiency, was found to be suffering from severe combined dual system deficiency disorder and pneumocystis. Administration of pentamidine ethionate, 4 mg/kg, was begun, and the patient was given large doses of transfer factor derived from a normal donor with strong PPD, Candida, and mumps cellular immunity. Within six days he manifested positive skin tests and MIF production concomitant with the donor's specificities. The blastogenic response, although not normal, appeared to show an elevated resting thymidine-incorporation level. The patient has recovered remarkably from his moribund condition, but the contribution of transfer factor therapy to this recovery, if any,

**TABLE 5. Results of transfer factor therapy of severe combined dual system deficiency patients (Under authors' protocol)**

| Patient (treated by) | Conversion of skin reactivity | MIF production | Comment |
|---|---|---|---|
| M.M.[a] (Hitzig) | Yes | Yes | Bone marrow transplant. Died of infection. |
| M.E. (Stiehm) | No | Not Tested | Bone marrow transplant successful. |
| B.W.[a] (Pirofsky) | Yes | Not Tested | Bone marrow transplant. Died of GVH. |
| E.D. (Levin) | Yes | Yes | Doing very well on repeated treatments with transfer factor and plasma infusions. |
| Summary | 3/4 | 2/2 | |

[a] Died.

is unclear. To date, 4 months after hospital discharge and receiving biweekly doses of transfer factor and plasma infusions, his chest is clear and he has had no new infections. This case will be reported in a latter communication.

This finding suggests that all patients with thymic alymphoplasia should be afforded a trial of transfer factor therapy prior to the institution of more radical reconstitution efforts such as thymic or bone marrow transplantation.

3. Autosomal recessive dual system deficiency—Swiss type (letter *D* on Figure 4). Autosomal recessive alymphocytic agammaglobulinemia is clinically and pathologically very similar to the above-described disorder. The major differentiating factor is the inheritance pattern (77–81).

To our knowledge there have been five patients with Swiss-type agammaglobulinemia treated with transfer factor: three with transfer factor provided by our group and two supplied by a French group (72). In those treated with our transfer factor, conversion of skin-test reactivity occurred in two out of three, and in both patients in whom it could be measured, MIF production also occurred. (One patient with X-linked recessive severe combined dual system deficiency also responded successfully to transfer factor therapy—See Table 5). One patient received a prescheduled bone marrow transplant after transfer factor was shown to be capable of inducing skin-test reactivity. This patient died of infection. One patient received a successful bone marrow transplant and is presently alive and well. A third patient converted to skin-test reactivity only transiently and could not be reinduced with the administration of one other dose of transfer factor. This patient was given a transplant of incompatible bone marrow and subsequently died of graft-versus-host disease. The results of transfer factor therapy in our patients are shown in Table 5. The results in these dual system deficiency patients have led us to reevaluate our thinking about the action of transfer factor. Perhaps transfer factor acts upon some cell types other than T-cells, for example the macrophage. Alternately it may act on precursor cells to permit them to differentiate into competent T-cells. The most reasonable possibility is, however, that these patients have some T-cell function, albeit minimal, which can be enhanced with transfer factor therapy.

*Variable deficiencies:* These include the following disorders.

1. Mucocutaneous candidiasis. The term mucocutaneous candidiasis describes a broad variety of disease states, all characterized clinically by candidiasis involving the skin and mucous membrane. Defects in cellular and humoral immunity have been reported among different populations of patients. Some patients have associated organ-specific auto-antibodies. Differences in response to specific therapeutic regimens and nonspecific immunologic induction regimens have been reported (82–85). This disease has also been associated with other forms of epithelial agenesis such as cartilage, hair, and fingernail aplasia (86).

Patients with this disease may have a wide variety of clinical and immunologic abnormalities. The process may be widely disseminated or localized. Patients may or may not show reactivity to Candida and other test antigens, and their lymphocytes may or may not respond in vitro to Candida or to phytohemagglutinin (PHA) and may or may not produce MIF. The disease can sometimes be brought into a lasting remission by the administration of amphotericin B, but it usually returns when antifungal therapy is stopped.

Successful therapy of mucocutaneous candidiasis with transfer factor used in conjunction with other agents has been reported (16, 87–89, 90).

At the time of this writing, we have heard a number of other reports of the successful use of transfer factor in patients with this disease, but these reports have not yet been published.

Our group has treated seven mucocutaneous candidiasis patients with transfer factor. The results are shown in Table 6. Detailed descriptions of these cases are reported elsewhere (90a). Briefly, our studies indicate two patients showing no response at all while the others showed varying minimal responses.

To date, results of therapy with lymphocytes or leukocyte extracts have been reported for 19 patients with mucocutaneous candidiasis. Clinical improvement was noted in four of these patients, but only one of those who improved did not receive simultaneous therapy with other anti-Candida agents, and in that patient the clearing was only partial.

2. Wiskott-Aldrich syndrome. The Wiskott-Aldrich syndrome is an X-linked recessive disorder characterized by thrombocytopenia, ezcema, and recurrent infections. The thrombocytopenia appears to be a result of an intrinsic platelet defect resulting in early senescence (91). Normal platelets injected into Wiskott-Aldrich patients have a near normal half-life (92), while Wiskott platelets have a shortened half-life when injected into normal subjects. Bone marrow aspirations reveal normal numbers of megakaryocytes. The recurrent infections are apparently caused by defects in both cellular and humoral immunity. The cellular immune defects can be measured by absence of skin reactivity and diminished in vitro parameters of cellular immunity; the humoral immune defect seems to be an inability to recognize certain carbohydrate antigens. There is a low level of serum IgM, often accompanied by a compensatory elevation in serum IgA. Recently it

**TABLE 6. Results of transfer factor therapy of mucocutaneous candidiasis**

| Patient | Candida Skin Test | | Lymphocyte Stimulation | | MIF Production | | Other Therapy | Result |
|---|---|---|---|---|---|---|---|---|
| | before | after | PHA | Candida | before | after | | |
| W.T. | – | – | Normal | Decrease | + | + | Clotrimazole | Dramatic improvement concomitant with clotrimazole therapy—transfer factor appears to play no part in improvement |
| P.T. | – | – | Normal | Decrease | – | – | Clotrimazole | Dramatic improvement concomitant with clotrimazole therapy—transfer factor appears to play no part in improvement |
| J.C. (Feigan) | – | +[a] | Normal | Normal | + | + | Amphotericin B | Improved |
| M.M. (Pirofsky) | – | + | Decrease | | – | + | Amphotericin B | Improved |
| S.F. (Hitzig) | – | + | Normal | Normal | + | | None | No significant change |
| L.Y.P. (Hitzig) | – | +[a] | Normal | Normal | + | + | None | No significant change |
| M.N. (Hitzig) | – | +[a] | Normal | | + | + | None | No significant change |

[a] Transient converson of skin reactivity.

has been reported that, when compared to children with "normal immunity" (cystic fibrosis) and recurrent pyogenic infections, children with this disorder have markedly lower levels of IgG, indicating a possible humoral defect in the IgG fraction as well (93). Some investigators believe that these patients are born with a normal complement of cellular immune constituents (94); however, due to the inability to recognize antigenic stimulation, these constituents rapidly atrophy and leave the child with a nonfunctional cellular immune system (T-cell system).

To date we have treated 11 Wiskott-Aldrich patients with transfer factor. The results of therapy are summarized in Table 7. Of these 11 patients, 6 responded to the transfer factor therapy. Freedom from infections occurred in 4 of 8, regression of splenomegaly was seen in 3 of 8, and clearing of eczema was seen in 5 of 8 patients. An unexpected clinical observation was improvement in the bleeding tendency in 3 of 8 patients which was associated with a slight rise in platelet counts in all patients. In one, it was also associated with regression of splenomegaly.

Of the 11 patients, 6 showed conversion of skin reactivity, and when it was measured, the lymphocytes of these patients also showed MIF production although they did not undergo increased DNA synthesis in response to antigen.

We demonstrated a similar dissociation between lymphocyte stimulation and MIF production using the tobacco mosaic virus protein (TMVP) system (95).

Guinea pigs which had been immunized with the whole protein showed skin reactivity and MIF production when tested with the antigenic peptides derived from the TMVP, but their lymphocytes did not undergo increased DNA synthesis in response to these peptides. These observations have led us to postulate that there may be two populations of thymus-derived lymphocytes or "T" cells, one of which responds to antigen by undergoing DNA synthesis and cell division, and a second which responds by producing MIF and perhaps other mediators of cellular immunity. Similar results were obtained in animals using a carbohydrate derived from the tubercle bacillus (96).

A deficiency in the monocyte IgG receptors was also found in some patients; this represents the second disease in which a monocyte defect has been found. The other is chronic granulomatous disease in which the monocytes have a defect in their ability to kill intracellular organisms, similar to that found in the polymorphonuclear leukocytes in this disease (97).

All patients with the Wiskott-Aldrich syndrome who had the defect in monocyte IgG responded to transfer factor therapy, while most patients without this defect did not respond. Only one patient who had normal monocyte IgG receptors responded to therapy with transfer factor. Other than the response to transfer factor, patients with the defect were clinically indistinguishable from those without the defect. We thus conclude that two or perhaps more pathogenic mechanisms probably underlie the disease. This is similar to the situation in hemophilia A and B, which clinically and genetically may appear indistinguishable, but which have different underlying defects.

**TABLE 7. Results of transfer factor therapy in the Wiskott-Aldrich syndrome**

| Patient | Improvement in infections | Conversion of skin reactivity | Regression of splenomegaly | Clearing of eczema | Lymphocyte stimulation | MIF production | Monocyte IgG receptor |
|---|---|---|---|---|---|---|---|
| J.P.[a] | Yes | Yes | Yes | Yes | No | Yes | Decrease |
| R.Z.[b] | No | No | No | No | No | No | Not Tested |
| K.C. | Yes | Yes | Yes | Yes | No | Yes | Decrease |
| K.N. | No | No | No | No | No | No | Normal |
| D.S.[c] (Pirofsky) | Yes | Yes | Yes | Yes | No | Yes | Not Tested |
| R.R. (August) | Yes | Yes | No | Yes | — | Not Tested | Decrease |
| C.T. (Stiehm) | No | No | No | No | — | Not Tested | Normal |
| T.R.[d] (Gatti) | None Present | No | — | No | Not Tested | Not Tested | Not Tested |
| D.H. (Hitzig) | — | Yes | — | — | — | — | Decrease |
| S.G. | — | Yes | Not present | ± | No | Yes | Normal |
| V.S. | No | No | No | Partial | No | No | Normal |
| Summary | 4/8 | 6/11 | 3/8 | 5/8 | 0/6 | 4/7 | 4/8 |

[a] Died—Chicken pox pneumonia—exposure to chicken pox during the waning phase of his eighth transfer factor cycle after 31 months of relative freedom from infections.

[b] Died—Leukemia, pseudomonas pneumonitis.

[c] Died—Amphotericin-B toxicity.

[d] Died—Cerebral hemorrhage.

Several considerations were of particular interest to us in the treatment of these patients. One was the specificity of the transfers. We have tried to determine in every case whether the transfer was specific or represented an increase in the immune response to all antigens. To accomplish this, we have performed the complete battery of six skin-test antigens on every donor and recipient of transfer factor. In one patient, J.P., we observed conversion of skin-test reactivity to one antigen (trichophytin) when the donor was negative. In another patient, D.S., the skin test to keyhole limpet hemocyanin (KLH) became positive after transfer factor (the patient had previously been immunized to KLH with negative results; the donor was not tested for sensitivity to KLH). In all other cases, the conversion of skin reactivity in the recipient was concordant with the reactivity of the donor. A number of recipients converted reactivity to only one or two antigens to which the donor was sensitive, usually mumps and/or Candida; we interpret this to mean that the sensitivity of the donor to the other antigens was insufficient to cause detectable reactivity. It has been recognized that the conversion of reactivity in the recipients depends on the degree of sensitivity of the donors to the antigens involved. We interpret the above findings to indicate that the action of transfer factor is specific rather than nonspecific.

Another consideration is the possibility of restoring T-cell function in the patients treated. It has been reported that both T-cell and B-cell functions are required for antibody formation in some antigen systems (98), whereas other studies indicate that for some antigens such as polymerized flagellin only B-cell function may be required (99). We were interested in exploring the possibility that transfer factor might restore T-cell function in these patients, which might then interact with normal B-cells, thus permitting them to mount a normal antibody response to carbohydrate antigens. Thus, we examined the isoagglutinin titers before and after the administration of transfer factor, but found that there was no significant change.

*Granulocyte defects:* These include the disorders outlined below.

1. Chronic granulomatous disease. This disorder was first defined as a clinical entity in 1957 (100). Patients have defective bactericidal capacity of polymorphonuclear leukocytes associated with failure of respiratory bursts and subsequent $H_2O_2$ production following microbial ingestion (101). Although the precise biochemical lesion is controversial, various groups have described NADH oxidase (102), NADPH oxidase (103), and glucose 6-phosphate dehydrogenase defects.

Neutrophils from these patients fail to reduce Nitro blue tetrazolium dye (NBT) when stimulated by polystyrene ingestion (104). They also have a significantly diminished intracellular bactericidal activity in in vitro testing (105). These patients suffer from recurrent pyogenic infections caused by a variety of pathogens, primarily staphylococcus aureus, gram-negative bacilli, and various fungi. Granulomas form in the liver; lungs and lymph nodes are filled with lipid-laden histiocytes. The pleomorphic genetic and clinical forms of this disease have suggested the more general term, neutrophil dysfunction syndrome, to describe this heterogenous group (106).

2. Job's syndrome. Job's syndrome appears to be very similar in both labora-

tory and clinical pictures to the above-mentioned disorder, but affects primarily redheaded females of Mediterranean descent. These patients suffer primarily from furunculosis or "boils"—hence the name.

3. Chediak-Higashi syndrome. Chediak-Higashi syndrome is a disease characterized by partial albinism and recurrent pyogenic infections. Large abnormal cytoplasmic organelles are seen in all granule-containing cells. These organelles are found in the nervous system, skin, ocular pigment, epithelium, peripheral nerve, hair, adrenal, and pituitary. Patients appear to have some lysosomal defect; however, they have normal NBT and killing tests (107). A recent report (108) indicates that the immunologic defect may be a quantitative one in leukocyte intracellular bactericidal activity.

Patients with the above-described granulocyte defects appear to have normal cellular immune activity as measured by presently available techniques. They may, however, have undetected defects, which would indicate that a prophylactic trial of transfer factor therapy may provide some clinical benefit. To our knowledge, no patient with granulocyte defects has been treated with transfer factor.

"*Acquired*" *immune deficiency states.*—These states can be divided into four major classes: (*a*) unknown, (*b*) infectious, (*c*) malignant, and (*d*) iatrogenic (see Table 4B).

Host responses in infectious diseases depend on (*a*) the virulence and pathogenicity of the invading organism and (*b*) the status of host resistance at the time of invasion. It appears that the "normal host" depends primarily on his cellular immune system for protection against slow growing and intracellular pathogens, while his humoral immune system protects him from fast growing, toxin-producing pathogens.

In many diseases like coccidioidomycosis, cellular immune reactions in the form of skin-test reactivity can be demonstrated shortly after the onset of clinical symptoms. Various types of antibodies directed against the invading organism can also be detected at this time. If the disease progresses and becomes disseminated the cellular immunity appears to diminish while the humoral immune component rises. This may be a result of the cellular immune system being overwhelmed, such as in antigen overload anergy. The reciprocal hyperactivity (albeit nonprotective) in the humoral immune system may be a result of increased antigenic exposure to the B-cell system or some homeostatic reaction.

The host, however, is protected from high-grade pathogens such as staphylococci, streptococci, and toxin-producing pathogens (diphtheria, clostridia, etc) by his humoral immune system. High titers of antibodies against these bacterial antigens appear to be protective. Cellular immunity against these pathogens can be demonstrated, and it may also play a major role in normal[4] host protection.

The host is protected from viral infections by both his humoral and cellular systems. During the viremia stage of the infections, specific antibodies can abort subsequent illness. When the virus becomes intracellular, the host's cellular immunity, through the elaboration of various antiviral factors such as interferon, limits the intracellular replication and spread of virus particles.

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[4] Patients with T-cell defects do have markedly increased susceptibility to bacterial infections. Therefore, the cellular immune system appears to play a role in host protection against these organisms, at least in some individuals.

In selected situations (such as neutrophil dysfunction syndrome and cystic fibrosis) where host cellular and humoral immunologic systems appear to be reasonably intact and some other facet of host resistance is defective, the clinical expression of these high-grade pathogens may be significantly changed. The pathogen which would normally be pyogenic may now change to low-grade pathogenicity and be attacked by the cellular immune system causing granuloma formation.

Immunologic reactions in malignancy will be discussed later.

*Diseases of unknown etiology with "acquired" immune deficiency:* Sarcoidosis is an inflammatory disease of unknown etiology characterized by the formation of noncaseating granulomas in many organs and tissues of the body. The pathologic changes are well documented in many texts. Sarcoidosis may result in the loss of cellular immunity to all antigens other than the sarcoid (Kveim). In other words, a patient who has a positive tuberculin test before developing sarcoidosis may lose his tuberculin reactivity as measured by skin-test reactivity, blastogenesis, and MIF production (109, 110). These patients develop cellular immune reactivity to the Kveim antigen (an extract of sarcoid spleen). The exact nature of this selective anergy is unclear and at present still controversial; however, this phenomenom may be a result of the patient's lymphoreticular system being overwhelmed or replaced by pathologic cells rendering it incapable of responding to other antigenic moieties. When patients with sarcoidosis are treated (paradoxically with immunosuppressant drugs such as steroids), they may redevelop their normal skin-test reactivity to tuberculin and candida.

*Infectious acquired immune deficiency states:* These states manifest themselves as variable deficiencies in both humoral and cellular immunity. For the sake of brevity, we will discuss only three disease states in which acquired immune deficiency plays a role: 1. tuberculosis, 2. coccidioidomycosis, and 3. leprosy.

1. Tuberculosis. The reciprocal of what obtains in sarcoidosis is true in tuberculosis. During the course of the disease, patients will first develop skin-test reactivity against the tuberculin antigen. If the disease becomes overwhelming, they will lose reactivity to the tuberculin antigen and may or may not maintain reactivity to other antigens such as Candida and other fungi. As the disease progresses, however, they may also lose reactivity to these other antigens. Again, the exact biochemical basis for this phenomenon is unclear; however, it is possible that the tubercle bacillus can render those lymphoid cells responsive against them tolerant (possibly on the basis of antigen overload anergy). As the disease progresses, the broad spectrum anergy may be a result of the total debilitation of the patient.

2. Coccidioidomycosis. Coccidioidomycosis is an infectious disease caused by *Coccidioides immitis.* The clinical disease is quite variable in severity and has cutaneous, bone, lymphoid organ, central nervous system, and pulmonary localization. Most patients will develop skin-test reactivity and precipitating IgM anti-

bodies against the coccidioides antigen shortly after the onset of clinical symptoms. If the disease progresses and becomes disseminated, there is a gradual diminution of cellular immune responsiveness against the coccidiodes antigen with an associated rise in the complement-fixing IgG-type of antibodies directed against that antigen. The exact relationship between these two phenomena is unclear; however, it appears that one of the systems of host resistance is overwhelmed and the remaining system may respond in a hyperactive way to overcome the overall immunologic deficit. An alternative hypothesis is that the continued presence of antigen may cause "exhaustion" of the cellular immune system (perhaps through impairment of production or function of the patient's own specific transfer factors) with a simultaneous increase in antibody production. If the patient is to recover from the disease state, a gradual return of cellular immune responsiveness with a diminution of humoral immune reactivity will be seen. The question of the possible role of these complement-fixing antibodies as "blocking factors" has been raised. This appears unlikely for two reasons: 1. the return of cellular immune competence and clinical recovery can be induced by specific antibiotic therapy which reduces the antigen load without any immediate direct effect on antibody titer, and 2. recovery of cellular immunity preceeds the reduction of antibody titer by 3 to 6 weeks in most cases.

3. Leprosy. Leprosy is an indolent infection caused by *Mycobacterium leprae*. Two forms of this disorder are recognized: (*a*) tuberculoid and (*b*) lepromatous. These variants may reflect differences in host susceptibility to the infection (111, 112). Tuberculoid leprosy occurs in the more resistant host and is principally characterized by symmetrical raised lesions of the skin and peripheral nodes which on biopsy appear to be granulomatous lesions. These patients generally respond upon skin testing with the lepromin (Mitsuda) antigen. In lepromatous leprosy, the disease begins with a raised red rash that is symmetrical and resembles that present in the tuberculoid type; however, the rash rapidly changes to nodular lesions in the dermis (lepromata) that vary in size. This form represents a more virulent infection and the prognosis is significantly poorer than for those with the tuberculoid form. These patients generally do not respond with skin-test reactivity to the lepromin antigen. In lepromatous leprosy, one often observes hyperglobulinemia with large quantities of antibodies directed against *M. leprae*. In the tuberculoid form, however, the patients have normal levels of immunoglobulins with significantly lower levels of specific antilepromin antibodies (113). High titers of anticollagen antibodies have also been reported in this disease (114).

Transfer factor has been used in patients with sarcoidosis. Urbach (115) was able to transfer local tuberculin sensitivities with the use of transfer factor. He was, however, unable to induce systemic sensitivity in these patients. Lawrence & Zweiman (17) confirmed these findings and also were able to develop feeble (10 mm) tuberculin reactions in test sites remote from the injection of transfer factor in two of seven well-documented Kveim-positive tuberculin-negative patients with sarcoidosis. Lawrence (116) feels that the tuberculin-type anergy of sarcoidosis is a result of antigenic competition with Kveim antigen which preempts the natural production or function of transfer factors for other delayed reactions as well as impairing the responsiveness of the patient's cells to injections of transfer factor produced by another individual. The results of the above-mentioned experiments indicate that dialyzable transfer factor can override the anergy induced by sarcoidosis and produce, albeit transiently, cellular immune reactivity to antigens other than Kveim. Transfer factor may be a very valuable tool when used in conjunction with more conventional modes of therapy in the treatment of sarcoidosis.

Transfer factor has been used in the therapy of coccidioidomycosis. One patient with miliary coccidioidomycosis and positive blood cultures was treated simultaneously with transfer factor derived from a donor who was strongly reactive to coccidioidin, and amphotericin B (117). Twenty-four hours after starting therapy, the patient developed a febrile episode with erythema nodosum. Lymphocyte transformation to coccidioidin converted from negative to positive. The patient's lymphocytes responded normally to phytohemagglutinin stimulation both before and after therapy. The clinical improvement was short-lived, however, and the patient died. The exact cause of death was undetermined since no autopsy was performed. These results corroborate the findings in sarcoidosis that transfer factor can override anergy and induce cellular immunity against antigens to which the host was previously anergic. This being the case, transfer factor therapy should be very valuable in the treatment of this disease.

Transfer factor has been used successfully to induce positive tuberculin and Mitsuda skin-test reactivity in previously anergic patients with leprosy (118). Several of these patients developed leproma reactions, which suggests some induced activity against leprosy bacilli. This study involved only one dose of transfer factor; therefore, clinical responses were not significant.

To our knowledge, transfer factor therapy has never been used in the treatment of tuberculosis. Since tuberculin reactivity can be readily induced in patients with other diseases, and since "antigen-overload anergy" can be preempted in the case of coccidioidomycosis and leprosy, it seems quite logical that transfer factor can induce tuberculin reactivity in the face of tuberculin anergy and possibly accelerate recovery.

Transfer factor should be used to treat overwhelming viral infections. Kempe (119) used viable whole lymphocytes to successfully reverse the course of vaccinia gangrenosa. Transfer factor would very possibly have been equally effective against vaccinia while not risking graft-versus-host or allergic reactions.

Another viral infection in which transfer factor therapy may be effective is herpes zoster. At our hospital a renal transplant patient with severe herpes zoster infection was treated with plasma from a donor who had recently recovered from variella infection but who had no demonstrable anti-variella antibodies. The patient responded dramatically to this procedure (120), and our group feels this response may have been mediated by specific transfer factor in the infused plasma.

When treating infectious diseases of any nature, the clinician must rely both upon the available chemotherapeutic moieties and host responsiveness. If the host is compromised and incapable of mounting resistance against an organism, an unlimited amount of antibiotics will not cure the illness. Most infectious diseases do not overwhelm host resistance and therefore are curable with antibiotic therapy; however, on those occasions when host resistance is overwhelmed, other therapeutic modalities should be considered. Transfer factor can enhance cellular immunity in patients who have acquired or congenital defects in cellular immunity, and controlled studies of its use in treating severe infectious diseases of all types in such hosts should be undertaken.

*Malignant "acquired" immune deficiency states:* Tumor protection in the normal host may be afforded by a cell-mediated "tumor surveillance" system which recognizes tumor-specific antigens on the malignant cell surfaces and destroys them before they develop into tumor masses. Defects in this surveillance system, whether congenital, acquired, or degenerative, may allow these cells to grow into tumor masses. In certain situations such as in congenital immune deficiency states, the immune deficiency disorder may be antecedent to and possibly related to the subsequent lymphoreticular malignancy. In lymphomas and reticulum cell sarcomas a defect in cellular immunity may be apparent. This may be a result of replacement of the normal cellular immune system by tumor cells or some specific depression of normal cellular immunity. These patients have an increased susceptibility to organisms which often infect patients with cellular immune defects, such as *Pneumocystis carnii* and fungal diseases.

In patients with malignancies of the myeloid system, such as granulocytic leukemia, the immune deficiency appears to be a lack of normally functioning myeloid cells. These patients are susceptible to a variety of organisms but particularly the high-grade pathogens, such as staphylococci and pseudomonas.

Malignant plasma cell disorders appear to induce several forms of immune deficiency in the patient. The replacement of normal immunoglobulin-producing plasma cells by malignant cells may reduce the normal immune response. An RNA-type of molecule which can passively transfer immunosuppression to non-tumor-bearing animals has recently been described (121). This mechanism may also render the myeloma-bearing human immune deficient.

The rationale for the use of transfer factor to increase cellular immune antitumor activity is outlined below.

In normal experimental animals, tumor immunity is relatively easy to achieve either by active or passive immunization. Thus, spontaneous tumor growth in the immunized animal can be significantly reduced and the immunized animal injected with tumor cells or virus may show no growth of the tumor. Immunotherapy of established tumors using immunocompetent cells has been less encouraging; however, there have been some reports of regression of established tumors achieved by the administration of sensitized cells. Transfer factor would not be expected to be effective in animal tumor systems since, as indicated above, it does not appear to be effective in experimental animals. Indeed the transferred cells must be viable to inhibit tumor growth (122).

Serum factors (blocking factors) have been shown, at least in vitro, to inhibit the reaction between sensitized lymphocytes and tumor cells, as measured by colony inhibition (123, 124) or ability to inhibit macrophage migration (125, 126). These factors may be related to "enhancing antibodies" which facilitate tumor growth (127, 128). This may explain the ineffectiveness of transfer factor and whole cells in the treatment of established tumors.

Certain animals undergoing tumor regression (129) or patients free of tumor (130) may also have a serum factor (unblocking factor) which inhibits the effect of the blocking factor, thus permitting the interaction of the sensitized lymphocytes and the tumor cell. Furthermore, infusion of serum containing this factor into animals bearing progressively growing tumors can cause regression of these tumors (129).

Serum from patients with osteogenic sarcoma has been shown by immunofluorescence (131, 132) and complement fixation to contain antibody to the donor's tumor. These antibodies are also present in a high proportion of the patient's family. Patients with malignant melanoma often have antibodies to their tumor as demonstrated by immunofluorescence or complement fixation (133–135). In these patients, the occurrence of a high titer of complement-fixing antibody appears to be correlated with a favorable course, and extension of the disease is associated with a reduction in antibody titer.

Patients have been skin tested with extracts of their own or other tumors, and about one fourth show skin reactivity to the tumor extracts (136–138). Furthermore, the occurrence of skin reactivity has been correlated with the occurrence of lymphocyte infiltration of the tumor nodules (138).

A number of investigators have studied the in vitro cellular response of patients to their tumors and have been able to show cellular immunity by lymphocyte transformation (139, 140), colony inhibition (141), leukocyte migration inhibition (142, 143), and macrophage migration inhibition (37, 125, 126, 144, 145). It is not known how the occurrence of in vitro reactivity correlates with the prognosis of the disease; in at least one patient we have studied there was very strong in vitro reactivity against antigens of the same type of tumor, and yet there was virtually no inflammatory response in the tumor metastases.

Southam (146) reviewed the early attempts at immunotherapy of cancer, which included attempts at active and passive immunization, attempts to nonspecifically stimulate host defenses, and the administration of nonspecific factors. For the most part passive immunization involved the administration of heterologous antibody to the tumor rather than transfers of sensitized cells. He concluded that there is no proof that any of these manipulations influenced the course of the disease in any patient. On the other hand, occasional clinical responses were observed, and these should not be disregarded.

That the delayed sensitivity reaction can play a role in causing tumor regression is demonstrated by the observation that the injection of tumor nodules with a substance such as PPD, SKSD (Streptokinase-Streptodornase), or DNCB which causes delayed reactions to occur over the nodule may cause regression of the nodules (147, 148). The regression caused by the injection of Bacillus Calmette

Guerin (BCG) or vaccinia into the nodule may be related to this mechanism, but may also heighten the patient's specific and nonspecific immune responses.

A large-scale study was conducted to achieve tumor regression by means of the transfer of homologous specifically sensitized lymphocytes using as donors pairs of patients with cancer (transplanted with the tumor of their partners) (149–151). Leukophoresis was performed three times a week, and leukocytes from the recipient of the tumor were infused into the tumor donor. In this way tumor regressions were achieved in some of the patients, and one of these regressions lasted more than four and a half years.

A similar approach using white cell lysates was performed by Thompson (152). The donors were sensitized by the repeated injection of irradiated tumor from the recipient. Forty patients have been treated to date. Of 19 patients with extensive metastases, 7 experienced objective clinical improvement (50% regression of measurable tumor), while 5 of 7 patients with minimal or no visible disease remained free of disease nine months after the start of therapy. Similarly, 2 patients with metastatic melanoma were treated and 1 obtained a partial remission (20–25% regression in size of skin nodules) (153). However, the patient died three weeks after the initiation of therapy from hemorrhaging into a cerebral metastasis. In another study (154), 5 patients with breast cancer were treated with transfer factor derived from normal donors. The rationale was that some of these healthy donors would have had cancer of the breast, but would have rejected it through the mechanism of "immunologic surveillance" and thus possessed immunity which could be transferred to the recipients. Doses up to the equivalent of 257 ml of packed leukocytes were administered over a period of up to 310 days. Apparently 1 of the 5 patients showed a temporary partial regression of her tumor. Three patients showed conversion of delayed sensitivity to tuberculin and/or streptococcal antigens. None developed antibodies to histocompatibility antigens or suffered allergic reactions. This study shows that large amounts of transfer factor can be safely administered with no untoward effects.

We have used transfer factor for up to 18 months in 7 patients with malignancy, and have treated patients with several different kinds of malignancy. We have used highly selected subjects as donors for the transfer factor and have given repeated injections to the recipients. We have evaluated immunity to tumor-specific antigens in the recipients before and after the administration of transfer factor, but it is too early to state the clinical results of these studies.

There are many problems in whole-cell therapy which should be considered. One is that patients are sensitized to histocompatibility antigens. This means that the patient would be specifically sensitized and donor leukocytes would be rapidly rejected. Donor transfer factors might be released during the rejection process and produce beneficial effects; however, we think this is unlikely. Dialyzable transfer factor is nonimmunogenic, so this problem is obviated.

Another problem associated with whole-cell therapy is the possibility of evoking enhancing or blocking antibodies in the recipient. Transfer factor therapy does not carry this hazard, since it does not induce antibody formation (155).

Transfer factor therapy may be effective in the treatment of leukemia. Evidence for the protective role of cellular immunity comes from various studies. Protection from spontaneous leukemogenesis in guinea pigs can be passively transferred using lymphocytes from previously immunized donors (156). Clinical parameters of cellular immunity—skin-test reactivity, rosette-forming cells, and blastogenesis—have been shown to wax and wane in temporal relationship to relapses and remissions of leukemia (157). Thus, cellular immunity may play a role in the induction of remission and protection from relapse, and its enhancement using transfer factor may be clinically advantageous.

Our goal in the use of transfer factor as a therapy for malignant disease has been the induction of the strongest possible cellular immunity in the patient, hoping that this might tip the balance in favor of tumor regression and against enhancing factors. Perhaps the use of specific transfer factor, in combination with chemotherapy and/or the use of nonspecific immunologic stimulants (BCG), will be the key to the successful therapy of cancer.

*Iatrogenic immunosuppressive therapy:* Immunosuppressive therapy has become a prominent treatment for patients with allogeneic transplants, "autoimmune" disorders, and malignant disorders. Many varieties of immunodeficiencies affecting both the humoral and cellular immune systems may be seen depending upon: (*a*) the drug used, (*b*) the state of host resistance of the recipient, and (*c*) the disease state for which the drug has been used. All variants of immune deficiency disorders can be seen in patients on immunosuppressive therapy.

## CONCLUSION

Transfer factor is a dialyzable leukocyte extract which transfers delayed hypersensitivity from a skin-test positive donor to a skin-test negative recipient. It is nonantigenic and contains no transplantation antigens. Therefore, under conditions in which it is effective, it is preferable to the use of whole lymphocytes or bone marrow, since these modalities carry the hazard of fatal graft-versus-host reaction in the immunologically incompetent host. Transfer factor appears to be effective in about half of the patients with the Wiskott-Aldrich syndrome, but the results of its use in mucocutaneous candidiasis have not been encouraging. Perhaps the most exciting field of exploration at the present time is in the therapy of malignant and infectious diseases. The chemical nature of the active substance in transfer factor is unknown, but its low molecular weight is well documented in many laboratories by the observation that it is dialyzable. The lack of adequate in vitro test systems or animal models hinders its further characterization. The widespread current interest in transfer factor will undoubtedly result in answers to at least some of the many remaining questions.

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# MENTAL EFFECTS OF L-DOPA

7071

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The psychoactive properties of L-3,4-dihydroxyphenylalanine (L-dopa) are of interest because behavioral changes represent a significant limiting factor in its use in treating patients with Parkinson's disease. In addition, as L-dopa is the natural amino acid precursor of the central neurotransmitters dopamine and norepinephrine, studies of the behavioral changes observed with L-dopa may provide a relatively direct means to assess the role of central catecholamines in human psychological functioning. This review considers the effects of L-dopa on cognition, mood, and behavior in man. Most emphasis is necessarily on the effects of L-dopa in the great number of parkinsonian patients who have received this drug; however, these effects will be compared with those observed in the smaller groups of patients with other neurologic and psychiatric disorders, as well as in the small number of normal controls treated with L-dopa.

Interpretation of the psychoactive effects of L-dopa in parkinsonian and other individuals is complicated by several factors: (*a*) the high incidence of psychiatric disorders in parkinsonian patients prior to treatment, especially organic brain syndromes and depression (1); (*b*) nondrug-related factors including the personal and social challenges brought on by the effective reversal of this chronic illness; (*c*) the concomitant effects of anticholinergic drug use in some studies, as these agents also have some psychoactive effects; and (*d*) the limitations inherent in the data, since most reports provide only descriptive summaries of the cooccurrence of behavioral changes with L-dopa treatment—in several instances validated measures of intellectual function have been used (2–8), but there has been only sparse quantitative investigation of other psychological and behavioral functions in placebo-controlled double-blind studies (9, 10).

## BEHAVIORAL CHANGES IN PATIENTS WITH PARKINSON'S DISEASE TREATED WITH L-DOPA

Several recent papers (11–17) and a book (18) provide up to date reviews of the more than 50 reports of the effects of L-dopa on parkinsonian patients. This material can only be summarized here, and references will be used only to document particular points. Behavioral changes reported as side effects of L-dopa administration in patients with Parkinson's disease include irritability, restlessness, anxiety, anger, agitation, hostility, outbursts of aggressive behavior, confusion, disorientation, delirium, paranoid ideation, delusions, hallucinations, hypersexuality, hypomania, depression, and suicide attempts. Daytime somnolence and nighttime insomnia, as well as vivid dreams to the point of nocturnal hallucinations have also been reported. In addition to these specific behavioral changes which occur only in some individuals, a general alerting or awakening effect of L-dopa associated with increased interest in the environment was first noted in many patients by Cotzias et al (19), and has been affirmed in other reports (20, 21). Its occurrence is not closely correlated with the degree of motor improvement, but rather has been suggested to be a separate phenomenon (17).

The incidence of specific psychiatric problems during L-dopa treatment ranges from 5–55% of patients treated, with an average incidence of serious symptoms being approximately 15% (11–17) A state of restlessness and agitation, and a confusional delirious state with some psychotic features are the two most frequent symptom clusters, each occurring in about 3–5% of all patients treated. Somewhat less frequent (1–3%) are depressive symptoms, paranoid or other psychoses, and hypomanic episodes. The great variance in incidence and the wide range of reported symptoms may result from differences in patient selection factors and diagnostic criteria. Age and pretreatment psychological and neurological status are of particular importance, since many studies have included patients with preexisting dementia, postencephalitic patients, and older patients in whom a higher incidence of arteriosclerotic changes may be expected. Behavioral side effects, particularly confusional states often accompanied by psychotic phenomena, are more common in patients with preexisting brain syndromes; it is primarily in this group that behavioral side effects may persist and require discontinuation of L-dopa treatment (22, 23). One parkinsonian patient without behavioral side effects could no longer tolerate L-dopa after suffering a head injury which produced no evident mental impairment except a recurrent hallucination which occurred only during L-dopa treatment (24). Other factors shown in some instances to affect the incidence of psychiatric symptoms include higher dosage (increased side effects), and length of treatment [some behavioral changes occur in the first months of treatment while others appear only after more prolonged L-dopa administration (25)].

Although most L-dopa studies only briefly discuss behavioral side effects, several reports focusing exclusively on behavioral changes provide more detailed information on individual responses (11–15). Celesia & Barr (11) studied 45 parkinsonian patients of whom 16 (36%) developed one or more psychiatric symptoms which were considered intolerable and which required medical attention during L-dopa treatment. Psychotic symptoms including delusions, hallucinations, reality distortion, and in some instances aggressive or assaultive behavior occurred in 8 patients. Of these, 6 developed an associated confusional state with disorientation and impaired judgment and memory; 5 of these latter 6 had a preexisting mild chronic brain syndrome with forgetfulness and concrete thinking but no previous history of psychotic behavior. The 2 patients without confusional symptoms constituted 2 of the 3 patients in the study with postencephalitic parkinsonism. Four additional patients with mild chronic organic brain syndromes developed increased confusion, disorientation, and memory impairment without psychotic symptoms. Euphoria or hypomania developed in 4 patients. Acute anxiety with apprehension, nervousness, and irritability occurred in 4 patients, while 2 individuals developed depressive symptoms treated successfully with imipramine. Most of the other psychiatric symptoms remitted with reduction of the L-dopa dosage, although discontinuation of the drug was necessary in 4 patients (9%). A close relationship between the occurrence of psychiatric symptoms and dyskinetic movements was suggested for these patients, although this has not been commonly noted in other studies.

Of 90 consecutive patients studied by Jenkins & Groh (12), 18 (20%) with an average age of sixty-six treated for a minimum of three months developed psychiatric symptoms considered related to L-dopa. Psychotic symptoms including hallucinations, delusions (often paranoid), excitement, and agitation occurred in 7 patients; in 14 patients depressive symptoms sometimes accompanied by suicidal activity became manifest. In addition, 22 patients reported impaired memory, 19 reported insomnia, 17 reported vivid dreams or nightmares, and 3 reported hypersomnia. In general, most of the behavioral side effects appeared to be dose-related and frequently improved with dose reduction; the depressive symptoms were treated successfully with tricyclic antidepressants. In contrast to the high incidence of depression in this study, O'Brien et al (13) observed a reduction in depression following L-dopa treatment in half of the patients who had pretreatment elevations on Beck and Hamilton depression scale ratings. A similar distribution of other psychiatric side effects was noted in this study and in those of Cheifetz et al (14) and Damasio et al (15), the latter presenting explicit examples of different arousal states produced by L-dopa.

Late-developing side effects have also been described during L-dopa treatment. Barbeau (25) originally reported a syndrome of subtle mental changes, a persistence of stereotyped movements and hypokinetic-akinetic episodes which occurred in greater frequency after two years of treatment. The mental changes consisted of a decrease in attention span, occasionally accompanied by a loss of recent memory, together with changes in social behavior, including a "frontal-lobe-like insouciance." Of 68 patients with a mean age of sixty-two who were treated continuously for over two years, 14 (21%) developed all or part of this syndrome.

## Behavioral Changes in Non-Parkinsonian Subjects Treated with L-Dopa

Detrimental behavioral effects have not been commented on in anecdotal reports of normal subjects receiving low doses of L-dopa (20, 26, 27), nor have significant behavioral side effects been specifically reported in patients with narcolepsy (28), impotency (29), or migraine (30) receiving L-dopa. In contrast, demented patients without Parkinson's disease were observed to develop restlessness and mental or motor agitation during L-dopa administration (31). In schizophrenic patients, L-dopa treatment was associated with agitation and increased delusions or hallucinations (32).

In a double-blind study (9), a small proportion of patients hospitalized for depression (25%) improved during L-dopa administration. A few suffered relapses during placebo substitution, although most patients remained depressed despite doses as high as 12 g/day. Nonimproved depressed patients developed increased anger and psychosis ratings on a quantitative behavioral scale, although the increased psychotic behavior occurred only in individuals who had pretreatment evidence of psychosis. Of 9 depressed patients with histories of mania (bipolar patients) 8 developed brief hypomanic episodes during L-dopa treatment (10). In contrast, unipolar depressed patients (those without a history of mania) did not develop hypomania during treatment with L-dopa in equal doses. The occurrence of hypomania was dose-related and was associated with relatively greater urinary dopa excretion in the bipolar compared to the unipolar patients (33).

## IQ, LEARNING, AND MEMORY CHANGES ACCOMPANYING L-DOPA TREATMENT

Parkinsonian patients receiving L-dopa not only exhibit improvement in motor function tests and the performance subtests of the Wechsler IQ scales, but also show approximately twice as much improvement in the verbal IQ scales as expected on a test-retest practice basis (2–7). Depressed patients also exhibit improvement in verbal learning and memory tests during L-dopa treatment, with the change attributable to altered long-term memory rather than immediate information recall (8). The occurrence of a flood of specific, time-locked memories ("incontinent nostalgia") during treatment (34, 35) suggests that L-dopa may activate some process of state-dependent recall for specific arousal-related memories or behavioral patterns [cf hypomania in bipolar depressed patients (36)] resembling the state-dependent learning paradigm (37).

## BEHAVIORAL EFFECTS OF L-DOPA IN ANIMALS

Only brief mention can be made here of the experiments indicating that in large doses L-dopa generally enhances motor activity and conditioned avoidance responding, and produces stereotyped movements and compulsive gnawing (38–41) as well as an EEG arousal response in rodents and other species, including primates (42). Lower doses of L-dopa in combination with peripheral decarboxylase inhibitors (39) or inhibitors of monoamine oxidase (MAOI) (38) also yield increased motor activity, sometimes accompanied by increased aggressiveness and rage in the case of L-dopa–MAOI combinations (43, 44). Biogenic amine depletion by reserpine, α-methylparatyrosine, and parachlorophenylalanine appear to sensitize the animal to behavioral stimulation by L-dopa (38, 40, 45); tricyclic antidepressants potentiate some behavioral effects of L-dopa, including aggressiveness and irritability (46), while the phenothiazines and butyrophenones antagonize L-dopa's stimulant effects (44), most likely by a direct receptor blocking effect (47).

## BIOCHEMICAL BASIS FOR THE BEHAVIORAL EFFECTS OF L-DOPA

Although three specific dopaminergic tracts have been defined in the central nervous system (the large nigrostriatal tract which is most closely related to motor functions; a mesolimbic tract of undefined function; and the tuberoinfundibular tract which has a role in pituitary function) (48, 49), their relation to behavioral regulation remains unclear. L-Dopa leads to marked elevations of brain dopamine with much smaller and variable changes in norepinephrine (38, 39); however, it appears that a good share of the dopamine formed from L-dopa is synthesized in nondopaminergic neurons (e.g. as evidenced by displacement and loss of serotonin from 5-HT neurons) (50). Thus, while the prevailing view remains that the antiparkinsonian effects of L-dopa are directly related to dopamine replacement (51), and, correspondingly, that very possibly its behavioral effects are a consequence of increased dopaminergic tract activity, it is also possible that some behavioral effects of L-dopa result from other mechanisms. Interference with cholinergic, serotonergic, and noradrenergic as well as other mechanisms, or changes in the balance between catecholaminergic and other neurotransmitter systems may result from L-dopa administration (40, 50, 52, 53). It is noteworthy that L-dopa produces enhanced locomotor activity even after the majority of catecholaminergic neurons have been selectively destroyed by 6-hydroxydopamine (54). Denervation-type receptor hypersensitivity to L-dopa and apomorphine develops after 6-hydroxydopamine treatment (54, 55) and may well be present in patients with Parkinson's disease. This phenomenon may contribute to the more frequent occurrence of behavioral effects in patients with Parkinson's disease. Thus, while L-dopa is a natural precursor of the catecholamine neurotransmitters, the interpretation of its behavioral effects in relation to its neurochemical effects remains controversial, and several different mechanisms may be involved in its mode of action.

## BEHAVIORAL ACTIVATION WITH L-DOPA

A common factor in the wide spectrum of behavioral effects produced in man and animals by L-dopa appears to be a state of heightened arousal or activation. This psychomotor activating or alerting effect of L-dopa may be seen as a beneficial dividend in some individuals, and may account for enhanced learning, IQ, and memory. In other individuals, uncomfortable restlessness, anxiety, and insomnia may occur. In still others, especially those with preexisting neurologic or psychiatric disorders, L-dopa may lead to the activation of specific maladaptive behavior (e.g. hypomania or schizophrenic symptomatology) or to disintegration and disorganization of behavior, culminating in a confusional state or psychosis.

The concept that L-dopa affects behavior via the regulation of psychomotor activation agrees well with recently developed views of the potential importance of the distribution of catecholamine-containing neurons in specific brain areas (48, 49). It has been suggested that individuals developing psychiatric symptoms may have a common hypersensitivity to psychomotor activation induced by L-dopa, and that the deleterious effects of some other drugs (e.g. the tricyclic antidepressants, monoamine oxidase inhibitors, and amphetamines) in some individuals may similarly be mediated by a catecholamine-related activation process (17). Hyperarousal has been suggested as an important component of

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# BLOOD LEVELS OF PARATHYROID HORMONE IN DISORDERS OF CALCIUM METABOLISM[1]

7072

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During the past three years, new information concerning clinical parathyroid physiology has been generated rapidly. In much of the recent research, radioimmunoassay of parathyroid hormone (PTH) has been the critical method. After the initial description of the assay in 1963, several years elapsed during which many attempts to develop reliable assays failed (1). Even in retrospect, these early difficulties are not readily explained. Problems that appeared formidable at first did not prove troublesome in the long run, but unsuspected problems emerged as experience was gained.

Three major problems engendered delay: 1. Production of high affinity antisera to bovine PTH was difficult. It is now known that many different immunization schedules can be successfully employed for antibody production. The species of animal used for immunization is not critical since several species have yielded useful antisera. The early difficulties in antibody production can be attributed, at least in part, to limited supplies of antigen. 2. Antisera possessing high affinity to bovine PTH cross-reacted poorly with PTH in human plasma or serum.[2] Bovine hormone is most commonly used as the immunizing antigen since it is readily produced in quantity and can now be obtained commercially. The problem of low affinity of antibovine PTH sera to human PTH persists, but many antisera with sufficient binding of human PTH have been prepared so that sensitive assays in man can be performed. 3. Unavailability of a human PTH reference standard for expressing assay results posed a problem that only recently has been recognized as illusory.

Investigations from several laboratories have shown that even if human PTH were available in sufficient quantity for immunization, labeling, and standardization of assay results, the primary difficulty in PTH assays and their interpretation—the heterogeneity of circulating PTH peptides—would not be resolved.

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[2] In this discussion, we will refer consistently to serum assays. It should be noted, however, that many investigators perform assays on plasma. Equivalent results have been obtained using either sample.

The present status of PTH assays is best reviewed against the background of new information concerning the chemistry, immunochemistry, and metabolism of PTH. In this presentation, we concentrate on data influencing the interpretation of the immunoassay in man. At the present time, there is unavoidable confusion about immunoassays of PTH because of divergent results reported from various laboratories. With the data currently being generated in many laboratories, it is likely that PTH assays will soon be better understood.

## BASIC CONSIDERATIONS

### CHEMISTRY

In 1959, improved extraction and purification procedures were applied to PTH by Aurbach (2) and Rasmussen (3). Combined with the Munson bioassay and, more recently, immunoassay techniques, highly purified preparations of bovine PTH were prepared in many laboratories (4–7). Phenol extracts and urea-hydrochloric acid extracts of glands served equally well as substrates for purification. Until recently, however, even the best preparations of PTH by standard purification procedures were contaminated with nonhormonal peptides (8). This problem delayed determination of the primary structure of the hormone, but once solved, progress was rapid.

Brewer & Ronan (9) and Niall et al (10) independently and virtually simultaneously determined that bovine PTH consists of a single polypeptide chain of 84 amino acids. In addition, two isohormones, also composed of 84 amino acids but differing from the predominant form of the hormone in some details, have been isolated from bovine parathyroid glands (11).

Although bovine PTH is the only form of the hormone that has been completely characterized so far, information concerning the structure of PTH in other species is rapidly emerging. Porcine hormone has been partially sequenced (12, 13). In comparison with bovine PTH, it contains one fewer residue each of aspartic acid, alanine, and phenylalanine, one rather than two residues of methionine, and no tyrosine. The sequencing of human hormone has been made difficult by the scarcity of human parathyroid glands. Data indicate that human and bovine hormone are similar in size and charge but differ in some important respects (14). Determination of the complete covalent structure of human PTH is of great importance for the understanding of human parathyroid physiology and especially for better understanding of radioimmunoassays. This problem is being pursued by several groups, and it is likely that the structure of human PTH will be known by the time this review is published.

The structural requirements for biologic activity have been partially determined. Synthesis of the N-terminal portion of the molecule, residues 1 to 34, has been achieved by solid state methods, and it has been found to be fully active, as evidenced by activation of adenyl cyclase from renal cortex, hypercalcemic action, and phosphaturic action (15, 16). Further studies have shown that a fragment containing residues 1 to 29 is biologically active; fragments 1 to 13 and 1 to 20 are completely inactive. The N-terminal residue alanine appears to be essential for biologic activity since a synthetic peptide containing residues 2 to 34 is inert (8). All of these data were derived from a study of bovine PTH; it will be interesting to learn the extent to which human PTH yields the same structure-function relationships.

## BIOSYNTHESIS

The discovery of proinsulin, the biosynthetic precursor of insulin, stimulated interest in possible precursors of other peptide hormones (17). Earlier studies, based principally on the immunologic differences between PTH extracted from glands and that appearing in tissue culture media, suggested that the 84 amino acid PTH peptide itself may be a precursor to a smaller secreted molecular species of PTH (18–20). Suggestive as these studies were, they did not exclude the possibility that cleavage occurred following secretion.

It has been known for many years that some extraction procedures yield from parathyroid glands more than one biologically active fraction, and detailed investigation of some of these fractions has been successfully carried out in the last two years. Cohn, Hamilton and associates have isolated and partially purified a peptide that is larger than the 84 amino acid PTH from bovine parathyroid glands (21, 22). This peptide increases serum calcium concentration in the rat bioassay. On a weight basis, it possesses one half to one third the biologic activity of native PTH, and it reacts with PTH antisera. Studies by Kemper et al (23) confirm and extend the possibility that this peptide is a biosynthetic precursor of PTH which may properly be called pro-PTH. It appears to be larger than native PTH by some 15 to 20 amino acids and has a molecular weight approximating 11,500.

## SECRETION

Although a precursor to PTH exists in parathyroid glands, it is generally agreed that the major form of glandular hormone is the 84 amino acid peptide. The question remains: in which form is PTH secreted into the circulation? This issue not only is of fundamental biologic importance but also affects interpretation of measurements of circulating PTH by immunoassay.

Much new information has been derived from the combined use of whole organ or slice cultures of parathyroids and immunoassays. Using an antiserum to porcine PTH, Arnaud, Tsao & Oldham noted immunologic differences between PTH in the incubation medium of parathyroid adenoma slices and PTH extracted from glands (18). PTH in the medium was immunologically similar to PTH in human hyperparathyroid serum. Sherwood, Rodman & Lundberg also reported immunologic differences between PTH in glandular extracts and PTH obtained from organ culture media (19), and demonstrated apparent conversion of glandular to medium PTH by incubation of labeled PTH with parathyroid gland homogenates. Since glandular PTH is larger than PTH in media, it was suggested that cleavage to the smaller peptide occurs before secretion.

Although the immunologic differences between glandular and circulating PTH have been abundantly confirmed in many laboratories, the original interpretation of these in vitro studies is probably erroneous. Habener and associates have provided strong in vivo evidence that PTH is secreted into the circulation in the form of glandular hormone (24). They analyzed blood in thyroid veins draining the parathyroids and noted that the PTH was identical to glandular hormone; PTH in peripheral blood, however, consisted predominantly of peptide(s) smaller than glandular PTH. These small peptides presumably arise as a consequence of metabolism. This interpretation is supported also by less direct evidence (25–27).

Some of the factors controlling secretion of PTH are well established. Based on in vitro as well as in vivo studies, there is general agreement that calcium ion concentration is the principal regulator of PTH secretion. High concentrations suppress secretion while low concentrations stimulate it (28–30). To a considerable extent, magnesium concentration influences PTH secretion like calcium (28). The details of these studies are beyond the scope of this review.

## RADIOIMMUNOASSAY

### TECHNICAL ASPECTS

Partially purified bovine hormone, the antigen most commonly used for immunizations, has been injected with or without adjuvant into a variety of species. Most investigators immunize guinea pigs; some have had good success with chickens. In our experience, roughly 1 out of 10 animals injected yields a suitable antiserum. Most of the sera harvested contain some anti-PTH antibody, but few contain antibody with sufficiently high affinity to PTH for immunoassay. Since most antisera can be used in high dilutions, a serum harvested from a successfully immunized animal can be used for years.

The affinity of some antisera produced against bovine PTH is high for bovine hormone but low for hormone contained in human sera. This is not surprising since bovine and human PTH are known to differ in amino acid composition (14). The NIH-MGH group of investigators showed that one of their widely used antisera (GP-1) possessed roughly 50% of the affinity for human as for bovine PTH (31).

Highly purified PTH is required for labeling with either $^{131}$I or $^{125}$I. The available preparations contain some contaminating peptides but not in quantities sufficient to interfere with assay procedures. The Hunter-Greenwood method of labeling is universally employed with some minor modifications (32) as it is simple, rapid, and effective. A variety of methods have proved successful in separating labeled hormone from inorganic iodide and from hormone damaged during the labeling process. Microfine precipitated silica has been particularly useful for this purpose and is the most widely used agent in the preparation of the final labeled product (33).

Chromatoelectrophoresis has been the standard method of separating antibody-bound from free hormone (34). In recent years this technique has been largely supplanted by Herbert's dextran-coated charcoal method (35). It is simple and rapid, and a detailed description of its adaptation to the measurement of PTH is available (36). Many other methods of separation have been used; each has advantages and limitations, but assay results are not qualitatively affected by the choice of separation methods.

The absence of a satisfactory reference standard of PTH to which measurement of unknown samples could be related makes interpretation of the present literature cumbersome. For reasons indicated in the next section, none of the standards used in immunoassays performed thus far is entirely satisfactory. If bovine hormone, human hormone, and PTH contained in hyperparathyroid sera yield superimposable curves when the bound-to-free hormone concentration is plotted as a function of varying amounts of unlabeled hormone, any one of these forms of hormone may be used as an acceptable standard.

## IMMUNOCHEMICAL HETEROGENEITY OF CIRCULATING PTH

Berson & Yalow, the originators of immunoassay methodology first pinpointed this key issue (37). While studying patients with various forms of hyperparathyroidism, they noted that the estimation of hormone concentration depended on the choice of antiserum. When dilutions of test sera were assayed using different antisera, nonparallel responses were noted. Furthermore, the rate of decline of immunoreactive PTH after parathyroidectomy varied greatly depending upon the antiserum used. Based on these observations, Berson & Yalow suggested that circulating PTH consists of more than one peptide and that various antisera recognized these peptides to differing degrees. This conclusion has been abundantly confirmed in the last two years.

The evidence that glandular and tissue culture PTH differ immunologically has been reviewed earlier. PTH in tissue culture media is similar to serum PTH. According to Habener's important contribution, the majority of PTH in the parathyroid venous effluent is intact glandular PTH (24). These studies also suggest that a peptide larger than glandular 84 amino acid PTH can also be detected in the parathyroid venous effluent. This may represent secreted prohormone.

It is generally agreed that the predominant form of PTH in the peripheral circulation is smaller than the 9500 mol wt glandular hormone (24, 26, 27): the best current estimates suggest a mol wt of approximately 7000. After fractionation of concentrated human hyperparathyroid sera, we have been able to detect not only immunoreactive glandular hormone and fragment with a mol wt approximating 7000, but also an additional fragment with a mol wt of 4500–5000 (26). This latter fragment has not been detected by others, presumably owing to differences in procedures. In our system, the three circulating fractions are immunologically different, and after parathyroidectomy for parathyroid adenoma, display different kinetic properties. What we identify as glandular hormone disappears rapidly from the circulation, in good agreement with previous reports on the disappearance of iodinated bovine PTH injected into rats (38). The smaller fragments, however, persist in the circulation for longer periods of time.

There is no direct published evidence revealing the biologic activity of circulating PTH fragments. Indirect evidence suggests that the majority of circulating immunoreactive PTH represents a portion of the molecule beyond the N terminus, which is the biologically active portion (39). The experience of others suggests that the 7000 mol wt species as well as glandular hormone is biologically active (40). Canterbury & Levey (unpublished observations) have recently tested for biologic activity the three fragments isolated by our fractionation procedure. Activation of renal cortical adenyl cyclase was used as a test system. The fraction identified as glandular hormone possessed the expected biologic activity. The 7000 mol wt fragment was biologically inactive. However, strong biologic activity was consistently demonstrated in the 4500 to 5000 mol wt fragment. These preliminary data must obviously be confirmed and extended. For now, the question of the biologic activity of PTH fragments in the peripheral circulation must be considered unresolved.

Another unresolved problem of great theoretical and practical importance is the site(s) at which metabolic cleavage of PTH occurs. As reviewed previously, it appears likely that cleavage occurs after secretion from the parathyroid glands rather than at the time of secretion. There is some evidence that the kidney is one site of PTH catabolism (41–43). More recent evidence indicates that the liver also contains a PTH-metabolizing system (40, 44).

## PROBLEMS IN INTERPRETATION

As increasing experience was gained in the application of immunoassays of PTH to the study of human disease, divergent results from various laboratories became increasingly evident and distressing. The differences recorded were not only quantitative but also qualitative. How could a specific assay of the same peptide yield wholly different results in reliable laboratories? The presence of multiple immunoreactive PTH fragments in the circulation reconciles most of the recorded differences, but at the same time creates new difficulties in the interpretation of assay results.

The various antisera used for PTH immunoassay contain a family of antibodies, each of which possesses varying affinities to various circulating fragments. Unfortunately, none of the antisera used for clinical investigation has been completely characterized with respect to affinity to PTH fragments. Even if the affinity characteristics of a particular antiserum were completely specified, the problem in interpretation resulting from incomplete information about biologic activity of various circulating fragments would remain. For these reasons, all data presented thus far using a PTH immunoassay must be interpreted with caution. It is likely that antibodies to specific fragments will be produced in the future. One will then be able to examine not only prevailing steady state concentrations of various fragments, but also their kinetic behavior in response to physiologic stimuli.

In a sense, then, this review could end at this point. One might reason that there is no point in reviewing uninterpretable data. The situation, however, is not this bleak. There are many areas in which different assays have yielded concordant results, and divergent results have often served as a clue for illuminating research. Finally, some changes of immunoreactive hormone concentrations permit straightforward interpretation despite the limitations of a particular assay system.

## CIRCULATING PTH

### Normal

When the immunoassay was first developed, difficulty was experienced in measuring immunoreactive hormone in normal subjects under steady state conditions (1, 45). With improved techniques there is now general agreement that immunoreactive PTH can be detected in the majority of normal subjects (36, 46–51). In some assay systems, normal levels can be invariably detected (46). An inverse relationship between serum PTH and serum calcium can be documented within the normal range of serum calcium (36, 50). This confirms by direct measurement in man a critical relationship that has been well established in other species (52).

In the earlier studies, no circadian rhythm of serum PTH could be demonstrated (53). More recently, however, two groups have documented a clear-cut nocturnal increase of serum PTH. In one study, serum PTH was lowest between 7:30 and 11:30 AM and then increased progressively to a high point between 11:00 PM and 3:30 AM (54). In the other study, serum PTH was remarkably constant throughout the daytime hours, began to increase at 10:00 PM, and reached a high point at 2:00 AM (55). The physiologic basis for the nocturnal rise of serum PTH remains unexplained; a decrease of ionized calcium, the only unequivocably established stimulus of PTH secretion, does not appear to explain it.

Serum PTH is decreased by intravenous calcium infusions that raise the serum calcium concentration above normal and is increased by EDTA-induced hypocalcemia (36, 45, 56). However, the rate of hormone disappearance with the induction of hypercalcemia in man is slower than the rate expected on the basis of animal studies (57). This observation was an early clue to the possibility that some antisera recognize PTH fragments that possess kinetic characteristics different from glandular PTH (56).

### Hypoparathyroidism

In the idiopathic as well as the postoperative form of this disease, serum PTH is immeasurably low. This tautologic observation is not trivial since specimens derived from hypoparathyroid patients provide a critical control for validation of the immunoassay. Clearly, the detection of immunoreactive material in their sera would indicate lack of specificity of the assay system. From a practical point of view, sera from hypoparathyroid subjects are valuable not only as a test of specificity but also as diluents in various assay systems.

## PSEUDOHYPOPARATHYROIDISM

In the original description of this syndrome, Albright et al suggested that it was characterized by end organ refractoriness to PTH (58). Recent studies have confirmed this view by showing that the characteristic increased excretion of 3′,5′-AMP in response to exogenous PTH does not occur in pseudohypoparathyroid patients (59).

Increased PTH has been reported in peripheral and thyroid venous blood (48, 59, 60). In one study of three patients, PTH decreased from high to nondetectable levels with an intravenous infusion of calcium (59). Our own studies (Reiss & Canterbury, unpublished) also show unequivocal elevations of immunoreactive PTH in sera of six patients with pseudohypoparathyroidism. Since we frequently obtained nonparallel assay responses on dilution of these sera, it is tempting to speculate that we were measuring PTH fragments that differ from those in the circulation of hyperparathyroid subjects. Complete fractionation of one serum (Canterbury, unpublished data) indicated that the profile of PTH fragments in the peripheral circulation was somewhat different from that of patients with primary hyperparathyroidism. The available data are obviously insufficient for any firm conclusions. The possibility that pseudohypoparathyroidism represents an instance of abnormal metabolism of PTH cannot be excluded until more information is available on the character and biologic significance of circulating PTH fragments.

## PRIMARY HYPERPARATHYROIDISM

*Steady state concentrations.*—The earliest recorded experience showed a considerable overlap between assays in normal subjects and in those with primary hyperparathyroidism (45). With improvement of labeling and purification techniques and with proper choice of antiserum, the separation of sera from normal and hyperparathyroid subjects has improved (49). From the beginning, our own experience has been unusual in that we have always obtained a clear separation (46), undoubtedly because of the affinity characteristics of our particular antiserum. We suspect that antisera possessing high affinity for glandular hormone but weak affinity for fragments could not easily discriminate between sera obtained from normal and hyperparathyroid subjects; antisera with high affinity for the fragments would be well suited to such a differentiation. Following this reasoning, one would also expect that antisera with high affinity for glandular PTH would be well suited for studies involving short-term secretory changes and localization of abnormally functioning parathyroid glands.

Since absolute PTH levels do not differentiate clearly between sera from normal and hyperparathyroid subjects in some assay systems, several investigators have related immunoassays of PTH to the prevailing serum calcium concentration (36, 50). When this is done, the normal inverse relationship between serum PTH and serum calcium often effects a clear separation: while serum PTH may be within the normal range in patients with primary hyperparathyroidism, it is usually high considering the mild to moderate hypercalcemia. One could use this normalization of PTH by reference to serum calcium as an indirect suggestion that circulating PTH fragments are biologically active, for if circulating PTH is normal in absolute terms, what raises the serum calcium in hyperparathyroidism?

*Adenoma versus hyperplasia.*—Preoperative determination of whether the surgeon will encounter a solitary adenoma or hyperplasia of several glands would be of great practical value. The problem is difficult for pathologist and clinician alike (61, 62). When only a single parathyroid gland is examined, the differentiating characteristics may not be discernible.

Our early experience indicated that adenoma and primary hyperplasia could be differentiated by combining radioimmunoassay with calcium infusion (63). In patients harboring a single adenoma, the induction of severe hypercalcemia by an intravenous calcium infusion was not associated with any change of serum PTH. By contrast, in patients harboring two or more hyperplastic glands, serum PTH was decreased during the course of a calcium infusion. We interpreted these results as showing autonomy of adenomas and responsiveness of hyperplastic glands. Failure of calcium infusions to suppress PTH in primary hyperparathyroidism due to an adenoma was also recorded in another study (47). These in vivo studies appeared to be confirmed in vitro by the demonstration that, in organ cultures, PTH secretion of adenomas was usually independent of the calcium concentration in the incubation medium (64). By contrast, when normal or hyperplastic glands are incubated, there is an inverse relationship between PTH and calcium concentration in the medium.

Recent data have cast considerable doubt on the autonomy of adenomatous parathyroid tissue. Potts et al have demonstrated consistent decreases of circulating PTH in all patients with primary hyperparathyroidism, regardless of histologic classification (65). Obviously, the difference of results obtained with calcium infusions cannot be ascribed to selection of patients or classification of disease. Instead, the difference is more likely to be attributable to the specific affinity of antisera used in the assays. In our fractionation studies of sera from patients with adenoma and hyperplasia before and during an intravenous infusion of calcium, the difference between these forms of disease were quantitative rather than qualitative (26). In particular, immunoreactive glandular hormone was suppressed rapidly and completely in patients with hyperplastic disease, while PTH suppressed slowly and incompletely in patients with adenomatous disease. Thus, it appears that primary hyperparathyroidism is manifested as a spectrum of morphologic and functional abnormalities. The correlation between morphology and function is not as clear-cut as we originally suspected, nor is it likely to be completely absent as has been recently suggested. Additional work is required to clarify the issue.

*Preoperative localization.*—Surgery for hyperparathyroidism may be very easy or extremely difficult. There is no way of predicting preoperatively whether the surgeon's task will be simple or formidable. Hence, any guidance in preoperative localization would be of great practical value. We first applied the radioimmunoassay to this problem by measuring peripheral PTH before and after massage of one or the other side of the neck (63). The initial experience was encouraging with correct localization of abnormally functioning glands in all but one of the 18 patients studied. Patients harboring abnormal glands on both sides of the neck showed increases in peripheral hormone concentration after massage of either side.

Reitz et al (66) refined the method of localization by measuring PTH in venous blood draining the neck and mediastinum. The site adjacent to the drainage of the abnormal parathyroid tissue had a PTH concentration severalfold that measured in other sites. Subsequent experience with this procedure has been disappointing in that only approximately 60% correct localizations are attained (67). Further development of this general approach has now been achieved with great success. Doppman has provided a detailed description of the thyroid venous circulation, which he studied by means of selective venous angiography (68, 69). By immunoassay of samples obtained directly from the thyroid venous effluent, successful localization has been obtained in essentially all patients studied (70). When the procedure is successfully performed, the PTH gradient between blood draining the abnormal gland and that from normal glands is convincing. Successful localizations have been achieved in hyperplasia involving glands located on both sides of the neck. Localization studies are particularly useful in patients having had previous unsuccessful surgery for hyperparathyroidism. Surprisingly, despite the distortion of the venous drainage caused by the previous surgery, localization has been achieved in five patients. Since catheterization of the thyroid veins is technically difficult and time consuming, the procedure will likely be reserved for difficult patients and particularly those having had previous unsuccessful surgery.

*Effect of parathyroidectomy.*—Immunoassays are particularly useful in evaluating the results of parathyroidectomy (49, 53, 71–73). It is often difficult to be certain that all of the abnormally functioning parathyroid tissue has been surgically removed. This may be particularly vexing in patients with hyperplasia in whom the extent of the required parathyroidectomy may be in doubt. Curative surgery invariably lowers serum PTH to normal or low values.

## ECTOPIC HYPERPARATHYROIDISM

The long-held suspicion that certain cancers secrete PTH or a PTH-like substance has now been confirmed by immunoassay. The earliest direct evidence, based on a complement fixation assay, was soon followed by immunoassays (74, 75). The production of PTH by some cancers has now been abundantly established. Surprisingly, cancer patients frequently show elevated levels of PTH even in the absence of hypercalcemia (45).

Roof and associates have provided convincing evidence that ectopic PTH differs from native PTH immunologically as well as biologically (50, 76). It now seems likely that there are many forms of ectopic PTH, some closely resembling native PTH and others greatly different. The important theoretical implications of this observation are beyond the scope of this review. The practical implications, however, deserve emphasis. It is often clinically difficult if not impossible to distinguish between primary and ectopic hyperparathyroidism. The biochemical hallmarks of the two diseases may be identical, and even thorough clinical evaluation may not detect a small cancer. In this circumstance, immunoassay of PTH may be decisively helpful (50, 77). Some antisera accomplish the differentiation clearly; others do not distinguish adequately between ectopic and glandular PTH (72). In this as in other aspects of application of immunoassay techniques, selection of antiserum is the critical issue.

## Renal Hyperparathyroidism

There is general agreement that immunoreative PTH is increased in all or nearly all patients with renal insufficiency (45, 72, 78–84) As Berson & Yalow first noted, extremely high levels of PTH may be encountered in chronic renal insufficiency (45). On the whole, PTH levels tend to be higher in renal than in primary hyperparathyroidism.

Variable correlations between severity of renal disease and PTH levels have been recorded. In some studies, there is no correlation (45, 82); in others there is a rough inverse relationship between renal function and PTH (80). In all studies, however, there is much variability in PTH levels for any given level of renal function. PTH levels are positively correlated with the severity of bone disease in some but not all studies (82, 84).

Hypocalcemia is undoubtedly the stimulus for renal hyperparathyroidism. In early renal insufficiency, there are convincing data that phosphate retention with a reciprocal depression of serum calcium triggers secretion of PTH (85). This may be viewed as an adaptive response since the mild hyperparathyroidism reduces the tubular reabsorption of phosphate and thus restores normal or nearly normal serum phosphorus and calcium concentrations (86). In this interpretation, calcium and phosphorous homeostasis are achieved at the price of some degree of hyperparathyroidism. The role of phosphate retention in renal hyperparathyroidism has been studied exhaustively by Slatopolsky. He documented that in experimental animals the characteristic hyperparathyroidism of renal failure can be prevented by use of low phosphate diets (87, 88).

These apparently orderly adaptive responses characteristic of early renal disease are completely disrupted in advanced disease. When severe renal failure supervenes, reduction of the serum phosphorus from high to normal or low levels effects only trivial increases of serum calcium and has little or no decrease of PTH. Furthermore, there is no clear relation between serum calcium and PTH (72, 82). It is evident that serum calcium, PTH, and the forms of metabolic bone disease encountered in advanced renal failure are the result of complex hormonal and nutritional interactions. Of these, abnormal vitamin D metabolism is probably the most important but not the only factor (89). Major progress now being achieved in unravelling the metabolism of vitamin D should soon shed light on this problem (90).

The increased PTH of renal failure can be suppressed by intravenous infusions of calcium, but the rate of hormone decrease differs between assays (72, 82). Hypercalcemia associated with severe osteitis fibrosa is a rather common and disabling complication of longstanding renal insufficiency. Severe hyperparathyroidism is the rule, and parathyroidectomy is generally indicated. This has been named tertiary hyperparathyroidism and was once considered to represent the transformation of responsive (suppressible) to autonomous parathyroid function. PTH assays have not clarified the question of autonomy in this clinical setting any more than they have in primary hyperparathyroidism. In some assay systems, PTH is always suppressed with calcium infusions (80); in others, PTH is suppressed only in some patients (81, 82). It again appears that these differences can be explained by the different affinity characteristics of the antisera.

A particularly difficult clinical problem is metabolic bone disease in patients on chronic hemodialysis. Fournier et al noted that patients dialysed against a calcium concentration of between 4.9 and 5.6 mg/100 ml had higher PTH levels than patients dialysed against a calcium concentration of 6 mg/100 ml (84). PTH was lowest in patients dialysed against a relatively high dialysate calcium and who concomitantly achieved a normal or low serum phosphorus concentration. Companion studies showed that reduction of PTH in patients on high calcium dialysis was associated with definite amelioration of the bone disease (91). Raising the magnesium concentration in the dialysis bath may also suppress PTH (92).

Immunoassay of PTH has already provided important new insights into the genesis and control of renal hyperparathyroidism. PTH assays have been applied in only a very limited way to the study of the involution of parathyroid function after successful renal transplantation (49). The extant data suggest that the rate of this involuntary process is slow but unpredictable (93). As assays become more specific, much additional information will be elicited. The distribution of PTH fragments in the circulation will be particularly interesting. There are already some indications that PTH metabolism may be abnormal in patients with renal insufficiency (26).

## MISCELLANEOUS CLINICAL CONDITIONS

*Rickets.*—Increased levels of PTH have been recorded in nutritional rickets. On treatment with vitamin D, PTH decreased as expected (94). In vitamin D-resistant rickets, low, normal, and increased levels of PTH have been reported (95–98). These conflicting results demonstrate once again differences in antibody affinities. In addition, vitamin D-resistant rickets probably represents a heterogeneous clinical entity and that different patient populations were sampled in these studies. However, sufficient information is available to indicate that PTH is at best a contributory but not a causative factor in the genesis of the phosphaturia that characterizes the disease.

*Pregnancy.*—Only one study has been published (99). PTH decreased during midgestation and thereafter increased to levels clearly above normal. PTH was normal in postpartum women, including those who breast-fed for prolonged periods. The systematic study of calcium and PTH interchange between mother and fetus is still in its early stages (100).

*Sarcoidosis.*—In the majority of patients with this disease who have been studied, serum PTH was immeasurably low (101). This suppression is observed in normocalcemic as well as in hypercalcemic patients. Therapy with steroid hormones restores PTH to normal. The basic observation of suppressed parathyroid function can be explained by current concepts implying that excessive sensitivity to vitamin D or abnormal metabolism of the vitamin result in increased absorption of calcium from the intestinal tract and bone.

*Idiopathic hypercalcuria.*—Increased PTH serum levels have been recorded in approximately two thirds of patients with this syndrome (102). Treatment with thiazide diuretics abolishes the hypercalcuria and returns serum PTH to normal. This interesting observation deserves further study and introduces a final note of caution in the clinical interpretation of PTH assays. Normocalcemic primary hyperparathyroidism has been described by several investigators (103). Evidently PTH assays will not distinguish between this entity and the reversible hyperparathyroidism of idiopathic hypercalcuria.

## Concluding Remarks

Despite the limitations of radioimmunoassays as evidenced by divergent results from various laboratories, the PTH assay has already established itself as a powerful tool in clinical investigation. Given the drawbacks of the presently available polyvalent antisera, the assay still provides useful clinical information in many circumstances. The discovery of PTH fragments in the circulation has opened up new approaches. When specific antibodies to glandular hormone and to fragments become available for study, more definitive interpretation and purposeful applications of assays will be possible.

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# INSULIN RECEPTORS[1]

7073

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Insulin modifies a variety of cell functions including glucose transport, protein synthesis, glycogen metabolism, and lipolysis. About two decades ago Stadie and co-workers first advanced the concept that insulin's biological effects are the result of its binding to some cellular constituent (1–3). However, only recently has it been demonstrated that the initial step in insulin action is the recognition and interaction of the hormone with specific molecules localized in the cytoplasmic membrane of sensitive cells. These unique molecules, referred to as receptors, can interact with insulin with a high degree of specificity and affinity, and can convey the existence of this interaction to other structures responsible for emitting biologically significant events.

Early studies on insulin binding to intact, isolated tissues have been reviewed (4, 5). More recent studies on the identification, characterization, and isolation of the insulin receptor of liver and adipose tissue have also been reviewed (6–8). Accordingly, the present article only summarizes the main findings of these and more recent studies.

## MEASUREMENTS OF RECEPTOR INTERACTIONS

The first evidence for insulin "binding" to hormone-sensitive tissues arose from the indirect studies of Stadie and co-workers (1) who observed that rat diaphragms incubated briefly with insulin and washed showed a persistent increase in glycogen synthesis. Similar observations were made with lactating mammary gland (2) and epididymal fat pads (3). Subsequently these and other investigators attempted to directly demonstrate binding of the hormone to striated muscle and adipose tissue using insulin labeled with radiosulfur or radioiodine (9–14). These studies, however, were hindered by the low specific activity of the labeled insulin; it was impossible to discriminate between specific binding to receptors and nonspecific, physical adsorption to other tissue structures (15). Other investigators, working with intact fat pads (16) or isolated adipose tissue cells (17), studied the disappearance of native immunoreactive insulin from the incubation medium. Unfortunately, in these studies it was difficult to distinguish between hormone binding and hormone degradation, and the contribution of nonspecific adsorption of insulin to cells was difficult to assess. Furthermore, it has recently been demonstrated that under most conditions only a very small fraction (about 5%) of the insulin in the medium is specifically bound to tissue receptors.

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The most recent studies have shown that direct demonstration of specific insulin-receptor interactions in hormone-responsive tissues depends critically on the use of biologically active insulin labeled with radioiodine at a specific activity of 500 to 2000 Ci per mmole, as well as on the availability of homogeneous, intact, or broken cell preparations, such as isolated adipose tissue cells (18) or liver cell membranes (19). Insulin labeled with chloramine-T (20) at an average of less than one atom of iodine per molecule and purified by adsorption methods (21) is biologically active (22). Furthermore, monoiodoinsulin separated from native insulin by ion-exchange chromatography is fully active biologically (23). The importance of using properly labeled and purified insulin has been stressed (22) since biologically inactive, "damaged" components exhibit a high degree of nonspecific binding to cells or membrane fractions.

Structures capable of specifically binding iodoinsulin are present in isolated fat cells (22, 24–27), fat cell membranes (28), liver cell membranes (29–31), and certain circulating blood cells (32, 33). The basic properties of the insulin-receptor interaction in these various tissues appear to be virtually identical. The binding is time-dependent and saturable with respect to insulin. The concentration of insulin at which half-maximal saturation occurs is about $10^{-10}$ *M* (22, 28, 31), and the maximal binding capacity is about 10,000 molecules of insulin per intact fat cell (22) and 0.1 pmole of hormone per mg of liver membrane protein (31). The rates of association and dissociation of the insulin-receptor complex, which can be determined independently, obey the kinetics of simple dissociable processes (22, 28, 31). The dissociation constant calculated from these rate constants is about $5 \times 10^{-11}$ *M*; a similar value is obtained independently from equilibrium data. The binding is stereochemically specific with respect to insulin and insulin analogs (28, 30). Native insulin competes for binding in a way expected from the biological identity of iodo- and native insulin; desalanine insulin is indistinguishable from native insulin; proinsulin binds to the receptor with an affinity about 20 times less powerful than that of native insulin; desalanine-desasparagine and desoctapeptide insulins show a further decrease in affinity; reduced insulin, and S-sulfonated and carboxymethylated chains of insulin do not compete for binding.

The insulin-receptor complex can be dissociated with acid, and the insulin released from the complex is similar to unused insulin in several physical properties, in its ability to interact with membrane receptors and antibody, and in its ability to stimulate glucose transport in isolated fat cells (22, 28, 34). Virtually complete dissociation of the cell-insulin complex can also be accomplished by a large excess of insulin antiserum, and the cells' ability to bind insulin after such dissociation is not impaired (22). Although virtually any tissue or subcellular fraction can inactivate insulin, it is possible to demonstrate equilibrium binding of physiological concentrations of insulin without significant inactivation in the incubation medium using intact fat cells (22), fat cell membranes (28), and crude liver cell "microsomal" membranes (31). Marked inactivation of the hormone, accompanied by impaired immunoreactivity and altered physical properties, occurs with liver cell membranes prepared according to Neville's method (34). The latter inactivation process affects native insulin and biologically inactive insulin derivatives such as desalanine-desasparagine insulin to the same extent; it shows lower affinity for insulin than does the process of binding, indicating that binding and inactivation are not related (34).

Other properties of the insulin-receptor interaction have been studied in fat cell membranes (28). Measurement of the rate constants at various temperatures indicate that binding is much tighter at lower temperatures because the decrease in dissociation rate is disproportionately greater than the decrease in association rate. Binding is virtually unaffected by the nature of the buffer used or by a number of different ionic species, heavy metals, or metal-complexing agents. The optimum $H^+$ concentration for binding occurs sharply at about pH 7.5. An abrupt thermal transition with irreversible inactivation occurs with a midpoint at 53°C. High ionic strength causes a dramatic (up to sixfold) increase in insulin binding; this probably results from the appearance (unmasking) on the membrane of new binding sites for insulin. These are kinetically indistinguishable from those normally exposed. The effects of high ionic strength appear qualitatively similar to those of digesting membranes with phospholipases (25). Treatment of the membranes with several protein-modifying reagents suggests that tyrosyl and possibly histidyl residues may be important in the binding interaction.

The relationships between insulin binding and biological activation have been examined in intact fat cells (22, 24, 25). Evidence that the high-affinity binding observed is indeed a reflection of interaction with a biologically significant structure includes: 1. the similarity in the concentration dependence of insulin binding and hormone enhancement of glucose oxidation; 2. the exact correspondence (measured by affinity) between the relative ability of insulin derivatives (e.g. proinsulin) to enhance glucose oxidation and to compete with iodoinsulin for binding; 3. the parallelism between binding and biological effectiveness of insulins from various species (30); and 4. the similarity of the consequences on binding and on biological activation of certain modifications of the cell surface which specifically alter the affinity of the receptor for insulin, such as digestions by proteolytic enzymes (24).

## SUBCELLULAR LOCALIZATION OF RECEPTORS

The ability of insulin-agarose derivatives to simulate the metabolic effects of insulin suggests the presence of insulin receptors on the cell's surface (35). The insulin-binding activity of intact fat cells is quantitively recovered in the particulate fraction of the cell homogenate (22). The virtually complete loss of insulin binding after digestion of intact cells by trypsin-agarose is not altered by homogenization, indicating that no intracellular structures exist which are capable of binding insulin (36). Differential centrifugation reveals that the insulin-binding activity of fat and liver cell homogenates is preferentially localized in the microsomal fraction of the cell (22).

The asymmetric positioning of the insulin receptor has been examined by comparing the binding of insulin to everted and inverted membrane vesicles prepared from fat cell ghosts by procedures similar to those used with erythrocyte ghosts (V. Bennett and P. Cuatrecasas, in preparation). Inverted vesicles do not bind insulin unless they are disrupted by sonication or phospholipase digestion. Insulin bound to receptors and trapped inside such vesicles is not easily released into the medium, indicating that insulin-binding sites are restricted to the external surface of the membrane and that physical inversion of the normal membrane orientation is not accompanied by a major rapid reorientation of these specific membrane proteins.

## Effects of Enzyme Digestions of the Cell Surface

Drastic digestion of adipose tissue cells with neuraminidase causes a fall in the basal rate of glucose transport and abolishes the effects of insulin on glucose transport and lipolysis (26). Such enzymatic digestion, however, has no effect on the total quantity of receptors for insulin or on the affinity of the insulin-receptor interaction. The dissociation of the biological and receptor-binding properties caused by neuraminidase digestion suggest that membrane sialic acid is not involved in the recognition function of the receptor, but may be involved in the receptor's ability to convey the binding interaction to adjacent structures.

Digestion of adipose tissue cells by neuraminidase followed by digestion by $\beta$-galactosidase, or simultaneous digestion by these two enzymes, in addition to abolishing the biological effects of insulin, causes a profound fall in hormone binding (6). These findings suggest that galactose groups are involved in the recognition function of the receptor and may be chemical constituents of this molecule.

Very mild digestion of fat cells by trypsin causes a selective fall in the affinity of the insulin-receptor interaction and a corresponding fall in the apparent affinity of insulin-stimulated glucose transport (24). This occurs under conditions where the total amount of receptor and the maximal insulin response are unaffected. More drastic digestion of fat cells with trypsin causes severe loss of insulin responsiveness and insulin binding (24, 27, 37, 38). Such digestion decreases the total amount of receptor available for binding (24). When adipose tissue cells are first treated with phospholipases, trypsin causes severe destruction of the receptor without effects on affinity (24).

Digestions of fat and liver cell membranes with phospholipases C or A cause a dramatic (three- to sixfold) increase in insulin binding (25). These changes appear to reflect an increase in the total binding capacity of the membranes. The new exposed binding sites are kinetically indistinguishable from those normally present in the membrane. If the cells are first digested with trypsin, subsequent phospholipase digestion still has major effects, indicating that these "buried" binding sites are inaccessible to macromolecules in the medium. Addition of exogenous phospholipids can partially reverse the effects of phospholipases, and various chemical compounds which interact with membrane phospholipids mimic the effects of these enzymes. Extraction of membrane lipids with various organic solvents causes effects similar to those of phospholipase digestion (25).

## EFFECTS OF INSULIN-AGAROSE DERIVATIVES

Insoluble insulin-agarose derivatives simulate various metabolic effects of insulin (35, 39–41), and, when used in affinity chromatography experiments, are able to selectively and reversibly trap fat cell ghosts and water-soluble insulin-binding macromolecules (8). Insoluble polyacrylamide bead-insulin derivatives (8), as well as soluble dextran insulin (8, 42, 43) and ferritin insulin (8), are also biologically active (8). There is evidence that these insulin derivatives may be acting through mechanisms different from those occurring with native insulin (8). It has been suggested (8) that the insulin molecule, while binding to the receptor, brings to the receptor vicinity a large foreign surface which can perturb the cell membrane and thus nonspecifically initiate a biological response. Interestingly, a variety of other agents or stimuli apparently unrelated to direct activation of the receptor can also simulate the action of insulin in fat cells. Such agents include polyamines, organomercurials, ouabain, polyene antibiotics, and various enzymic digestions (8).

## RECEPTORS IN INSULIN-RESISTANT STATES

The possibility that metabolic states characterized by insulin resistance may be associated with derangements of the insulin receptor has been examined. The number of insulin-binding sites per cell as well as the affinity of the receptor sites appear to be normal in rats that are starved, treated with prednisone, or made diabetic by administration of streptozotocin, and in other species which show decreased responsiveness to insulin (44). Large adipocytes from adult obese rats also have normal binding capacities and affinities for insulin (45). The possibility that insulin resistance in obese rats might be explained by a decrease in the cell-surface receptor density has been discussed (8). A decrease in insulin binding to liver cell membranes has been observed in the obese-hyperglycemic syndrome of the mouse, a recessively inherited trait characterized by marked obesity and insulin resistance (46). The alteration in insulin binding appears to be explained by a decrease in the total number of receptor sites available rather than in the affinity of these sites for insulin (46).

## INSULIN RECEPTORS AND PLANT LECTINS

It has recently been demonstrated that the plant lectins, concanavalin A and wheat germ agglutinin, have marked insulin-like properties on isolated fat cells (47). Very low concentrations of these proteins are as effective as insulin in enhancing the rate of glucose oxidation and in inhibiting epinephrine-stimulated lipolysis in fat cells. In addition, these plant lectins can interact directly with the insulin receptor of fat cells in a way markedly perturbing to the insulin-receptor interaction (48). Low concentrations of wheat germ agglutinin enhance the apparent affinity of the insulin-receptor interaction in liver and fat cell membranes. Higher concentrations of this plant lectin, as well as concanavalin A, competitively displace the binding of insulin to receptors in these tissues. Such effects are equally apparent with insulin-binding macromolecules solubilized from membranes, indicating that the plant lectins directly interact with insulin receptors. The properties of the lectin-cell interaction have been studied in detail with iodinated derivatives of these proteins (49).

## INSULIN, MITOGENS, AND CELL GROWTH

It has been suggested that some of the biological effects of the plant lectins, such as their mitogenicity toward lymphocytes, might be related to the ability of these proteins to interact with insulin receptors and/or to initiate insulin-like biochemical events (47, 48). These considerations may be applicable to other growth-promoting hormones or factors, such as insulin, nerve growth factor, and somatomedin. It has recently been demonstrated that somatomedin ("sulfation factor," "thymidine factor"), the presumed mediator of growth-hormone action, has very potent insulin-like properties in a variety of target tissues and can interact directly with insulin receptors of fat cells, liver cell membranes, and chondrocytes (50). Like insulin, the plant lectins concanavalin A and wheat germ agglutinin (47), as well as somatomedin, (G. P. E. Tell, J. J. Van Wyk, and P. Cuatrecasas, unpublished), can inhibit the activity of adenylate cyclase in various isolated membrane preparations.

It has been demonstrated that human peripheral circulating lymphocytes possess no significant receptors for insulin (33). However, following stimulation by the mitogen, concanavalin A, there is a dramatic appearance of surface receptors the time course of which coincide with cell transformation. There is evidence which suggests that transformation may be related to decreased activity of adenylate cyclase. Lymphocytes permanently maintained in tissue culture as well as lymphocytes from patients with acute lymphocytic leukemia have about the same number of insulin receptors as are found in transformed lymphocytes (33).

## ISOLATION AND PURIFICATION OF THE RECEPTOR

The insulin-binding macromolecules can be quantitatively extracted in soluble form from liver and fat cell membranes with non-ionic detergents such as Triton X-100 (51). The kinetics and specificity of the interaction of insulin with the extracted receptor are similar to those observed in the original particulate system (51). The binding interaction is not affected by NaCl, neuraminidase, or phospholipase digestion (36). It is totally suppressed by concentrations of trypsin too low to affect binding in particulate fractions (36). Some physicochemical parameters of the binding macromolecule have been determined (36). Gel filtration and density gradient centrifugation experiments indicate a Stokes radius of 70 Å and a sedimentation coefficient of 11S. These parameters are unaltered by phospholipase digestions and delipidation of the membranes before solubilization. It has been calculated that the receptor has a molecular weight of about 300,000, a frictional ratio of about 1.5, and an axial ratio of about 9.

Purification of the soluble insulin-binding macromolecules is complicated by the continual requirement for detergent for maintenance of solubility and the extraordinary scarcity ($10^{-4}\%$ of the protein of a rat liver homogenate) of the binding protein in biological tissues (52). About sixtyfold purification can be achieved by ammonium sulfate precipitation followed by ion-exchange chromatography (52). Several insulin-agarose derivatives have been synthesized that can selectively adsorb, in affinity chromatography experiments, the insulin-binding protein from the detergent extracts of the membranes (52). Combination of these various procedures affords a purification of the receptor of about 250,000, which approaches theoretical purity (52).

## MECHANISM OF INSULIN ACTION

Under certain conditions insulin can decrease the concentration of cyclic AMP in liver and adipose tissue cells, and these changes appear to correlate with several biological effects (8). In liver and fat cell membrane preparations, insulin at physiological concentrations can inhibit the activity of adenylate cyclase, which is stimulated by epinephrine, glucagon, and fluoride (53–56). These direct effects of insulin on an enzymatic system in broken cell preparations suggest a fundamental role of adenylate cyclase activity and cyclic AMP in the mechanism of action of insulin. The possible relationships between the modulation of adenylate cyclase by insulin and the biological effects of this hormone have been discussed (8).

## CONCLUSIONS

Recent advances in the identification, isolation, and purification of insulin receptors provide new insights into the mechanisms of insulin action and provide a rational background for studying more detailed molecular events as well as relevant physiologic and clinical states. Moreover, the techniques and approaches which have been used may be generally useful in studies designed to elucidate the nature of the interactions of other peptide hormones or regulatory molecules with cell membranes.

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# ENDOGENOUS CARBON MONOXIDE METABOLISM[1] 7074

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It has now been over 20 years since the first demonstration that CO is endogenously produced in normal man (Sjöstrand 1). Methods of accurately measuring CO production in vivo and in vitro have been developed and the field has moved rapidly in recent years. Claims have even been made (Landaw 2) that endogenous CO production from man may contribute significantly to air pollution.

Sjöstrand's original data which suggested that CO originates from the alpha methene carbon atom of heme (Sjöstrand 3) have been rigorously confirmed. It is of interest that his in vitro experiments assumed Lemberg's original postulate that heme is degraded by coupled oxidation with ascorbate, a reaction which gives choleglobin which when acidified is converted into bilirubin. Ludwig, Blakemore & Drabkin (4) also utilized this reaction in studying degradation of hemin and demonstrated a molar ratio between hemin degradation and CO production. More recently it has become evident that heme degradation in vivo is not simply a coupled oxidation. Petryka, Nicholson & Gray (5) showed that coupled oxidation of hemin results in a variety of bilirubin configurations whereas bilirubin found in vivo is all or nearly all in the $IX^{a}$ configuration indicating cleavage of the ferriprotoporphyrin ring of heme only at the alpha position. It is now known that heme catabolism is an enzyme-catalyzed reaction (Tenhunen et al 6). In vivo isotope experiments in animals (Coburn et al 7, Landaw et al 8) and experiments where known quantities of chemically induced spherocytes were injected into normal human subjects (Coburn et al 9) have confirmed the nearly molar production of CO during heme catabolism and the theory that CO originates from the alpha bridge carbon atom of heme. It is a scientific curiosity that coupled oxidation of hemin gives similar byproducts and stoichiometry to in vivo heme catabolism. Actually, CO production from blood can be demonstrated under a variety of different conditions: heating, alkalinization (Coburn et al 10), etc.

## METHODS OF MEASURING RATE OF CO PRODUCTION ($\dot{V}_{CO}$)

A number of methods of measuring $\dot{V}_{CO}$ have been devised. In our opinion the rebreathing system (Coburn et al 11), where CO excretion is effectively prevented and CO production estimated from the rate of increase in venous blood carboxyhemoglobin percent saturation (COHB), has the fewest problems in interpretation of data. The method assumes that there is a constant relationship between blood COHB and the total body CO stores. This infers that COHB is homogenous throughout the circulation and that the partition of CO between blood and extravascular stores is constant. The method also assumes that CO is not lost from the body under conditions of rebreathing by metabolic or other processes. These assumptions have been partially tested in dogs and in normal man (Luomanmäki & Coburn 12, Coburn 13). The body CO "pool" mixes rapidly and can probably be considered one pool for the purposes of the CO measurement. It has been shown that the relationship of COHB to total body CO stores is constant over the COHB range seen during the $\dot{V}_{CO}$ measurement. Between 10 and 20% of the body CO is located extravascularly, primarily in skeletal and cardiac muscle bound to myoglobin (Coburn & Mayers 14). The possibility that blood COHB is influenced by changes in the partition of CO between blood and extravascular stores during the CO measurements has been excluded at least during the usual conditions in human and animal studies (Luomanmäki & Coburn 12). Under conditions of a very low arterial oxygen tension (<40 mm Hg) or hemorrhagic shock (12, 14, 15), large quantities of CO shift out of blood into skeletal and cardiac muscle, probably as a result of a decrease in intracellular oxygen tension in these tissues and the resultant increase in affinity of myoglobin and possibly cytochrome oxidase for CO (Coburn & Mayers 14). Thus, measurements of $\dot{V}_{CO}$ which are based upon determinations of COHB during rebreathing should be examined for the possibility of artifacts due to CO shifts between blood and muscle if the determinations were made under conditions of marked hypoxia in skeletal or cardiac muscle.

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It is known that CO is metabolized to $CO_2$ in normal man and various experimental animals, but the rate of loss of body CO by this route appears to be less than 5% of the rate of endogenous CO production under conditions of normal blood COHB in normal man, and thus is not significant. In dogs the metabolic rate of CO to $CO_2$ appears to be a function of blood COHB (tissue $P_{CO}$), so that at increased levels the rate of metabolic CO consumption becomes a more significant fraction of $\dot{V}_{CO}$. [The rate of metabolic consumption equaled $\dot{V}_{CO}$ at COHB of 10–15% (Luomanmäki & Coburn 12)]. Therefore, $\dot{V}_{CO}$ cannot be measured by the standard rebreathing method under conditions of elevated COHB. The biochemistry of CO oxidation has been reviewed recently by Fenn (16). It is possible that a disease state or states will be found where metabolic consumption of CO is increased.

The rebreathing method can measure $\dot{V}_{CO}$ under conditions where the relationship of venous COHB to the total body CO stores is varying due to shifts of CO between blood and muscle, or under conditions where metabolic consumption is significant, if the body stores are labeled with $^{14}CO$. $\dot{V}_{CO}$ can then be computed from the rate of increase in the ratio $^{12}CO/^{14}CO$ in venous blood during rebreathing (Wallace et al 17).

Normal $\dot{V}_{CO}$ values are available for young male subjects (11, 18–20), premenstrual female subjects (Delivoria-Papadopoulos et al 21, Lynch & Moede 19), and newborn babies (Maisels et al 22). Data from young normal male subjects obtained in four different laboratories are shown in Table 1. $\dot{V}_{CO}$ in young male subjects is about 15 to 30% larger than can be explained by destruction of hemoglobin in senescent erythrocytes, assuming a mean life span of 120 days. There is evidence that there is a daily and also weekly variation in $\dot{V}_{CO}$ in normal young male subjects which could not be explained by analytical error (Lynch & Moede 19). The mean variation in one day in any given subject was 16% (SD 13.4) and the mean weekly variation 32.5 (SD 17.8%). One might question whether this variation was actually due to endogenous production but the finding that in any one subject $\dot{V}_{CO}$ correlated with serum unconjugated bilirubin and also serum Fe concentrations is strong evidence that this variation actually resulted from variation in the rate of heme catabolism.

TABLE 1. CO PRODUCTION IN HUMAN MALE SUBJECTS

| Authors | Mean Age (yr) | Mean Weight (kg) | Rate CO Production [$\mu M$(24 hr)/m$M$ TBH[a]] |
|---|---|---|---|
| Coburn et al (11) | 31.2 | 72.2 | 10.4 |
| Coltman & Dudley (18) | | | 11.9 |
| Lynch & Moede (19) | 23.0 | 74.7 | 12.0 |
| Lundh et al (20) | 28.0 | | 9.9 |

[a] TBH = Total body hemoglobin computed from CO dilution measurements.

A different type of $\dot{V}_{CO}$ variation has been found in premenstrual females. Delivoria-Papadopoulos et al (21) found higher $\dot{V}_{CO}$ values during the progesterone phase of the menstrual cycle (22.4 $\mu M$ 24 hr/m$M$ TBH where TBH is equal to total body hemoglobin) than during the estrogen phase (11.5 $\mu M$ 24 hr/m$M$ TBH), a finding which was confirmed by Lynch & Moede (19) (17.6 and 10.4 $\mu M$ 24 hr/m$M$ TBH respectively). Administration of a progesterone derivative to normal male subjects resulted in an increase in $\dot{V}_{CO}$; therefore, it is suspected that progesterone is involved in the menstrual $\dot{V}_{CO}$ variation in females. There is evidence that progesterone may induce microsomal heme enzymes which when degraded resulted in CO production (21).

Recently the rebreathing method has been modified (Logue et al 23) so that changes in blood COHB are computed from infrared measurements of CO tension in rebreathing gas, using the Haldane Equation. This requires that mean pulmonary capillary oxygen tension and oxyhemoglobin percent saturation be assumed. Mean $\dot{V}_{CO}$ in normal male subjects was 15.9 $\mu M$ 24 hr/m$M$ TBH, a value considerably greater than that found in the studies shown in Table 1 where increases in COHB were determined with direct blood measurements (both infrared and gas chromatographic methods); the cause of this discrepancy remains to be determined. To be able to measure the low CO tensions in rebreathing gas with the desired precision it was necessary to increase the oxygen tension in the system to a value in excess of 600 mm Hg; it is possible that this caused erythrocyte membrane damage and increase in the rate of hemolysis (Coburn et al 24). With a long period of breathing in the hood subjects may gradually increase alveolar ventilation, thereby increasing alveolar and pulmonary capillary oxygen tension which will result, according to the Haldane Relationship, in an increase in CO tension. Increase in CO tension in the rebreathing gas may have been overestimated due to buildup of other hydrocarbon gases to which the infrared meter is sensitive. If there is no artifact in this study, the implication is that there is a group of normal human subjects in whom about one half of CO production does not originate from catabolism of RBC hemoglobin.

Rates of endogenous CO production have also been determined by measurement of the rate of CO excretion from the lung (Wranne 25). A basic problem is that it is practically impossible to reach a steady state between CO production, blood COHB, and pulmonary CO excretion (Coburn et al 24). The measurement could be accurately made if inspired CO is known and constant, and if alveolar ventilation, CO diffusion capacity, and alveolar $PO_2$ held constant for 15–20 hrs, the time required to approach a steady state. A workable method is to combine measurements of pulmonary CO excretion with measurements of changes in body CO stores. In this method the errors of the rebreathing system measurement are added to the considerable error in the measurement of expired CO. Under conditions where inspired CO varies during the measurements it becomes very difficult to achieve the desired precision.

It has been generally accepted that using the measurement of venous COHB as an index of $\dot{V}_{CO}$ leads to large errors due to the variation in the parameters which determine excretion of endogenous CO via the lung and the variable exposure to exogenous CO (Coburn et al 24). Engel et al (26) have suggested that venous COHB may be a better index of rate of hemolysis if it were compared with COHB of a normal adult or sibling exposed to the same environment. In their series, blood COHB from children with known hemolysis ranged from 0.8 to 4.0% saturation, whereas levels in normal children varied from 0.6 to 3.9% saturation. Blood COHB were almost always higher in the sibling with hemolytic anemia (average difference 0.2% saturation). It would be of interest to determine COHB differences between normal siblings.

## CO PRODUCTION AND HEME CATABOLISM

A major finding in the field of heme catabolism has been the characterization of microsomal heme oxygenase (MHO) by Schmid, Tenhunen and co-workers (27–30). Enzymatic conversion of heme to biliverdin in the rat liver was localized to the microsomal fraction and shown to be dependent on molecular $O_2$ and NADPH, and was inhibited by CO, suggesting that a mixed function oxygenase system was involved. The terminal oxygenase has been identified as cytochrome P-450. This reaction is coupled to a soluble NADPH-dependent biliverdin reductase giving bilirubin. It was demonstrated that most or all of the bilirubin formed was in the $IX^{a}$ configuration and that equimolar production of CO and bilirubin occurred. The most active substrates were (in order of decreasing activity) protohemin IX, methemalbumin, methemoglobin, alpha or beta chains of hemoglobin, deuterohemin IX, coprahemin, hemoglobin-haptoglobin. Myoglobin, carboxyhemoglobin, oxyhemoglobin, and protoporphyrin are not substrates for this reaction. MHO activity in the rat (expressed per unit wet weight) was highest in the spleen followed by bone marrow, liver, brain, kidney, and lung. Activity of this system was increased in rat liver following injections of hemoglobin, methemoglobin, or methemalbumin, and following experimental hemolytic anemia. It is unclear whether the mechanism of this increase in activity is an increase in the number of active cells, activation or stabilization of enzyme, de novo synthesis, or allosteric changes. Bakken et al (31) have shown that MHO activity in rat liver increased following epinephrine or glucagon injection. Furthermore, this increase in activity was associated with a fourfold increase in CO production, implying that the rate of hepatic heme catabolism is influenced by activity of this enzyme system.

It is convenient to divide processes which result in bilirubin and CO production into two groups based on appearance of $^{14}$C-bilirubin and $^{14}$CO following injection of glycine-2-$^{14}$C. The "early labeled peak" appears to originate principally from degradation of hepatic cytochromes and unassigned heme which may serve as a precurser in formation of hepatic hemoproteins. A small erythropoetic component lies buried in the descending limb of the early labeled bilirubin curve. The second group is demonstrated much later following glycine-2-$^{14}$C injection with $^{14}$C-bilirubin and $^{14}$CO arising as a result of hemoglobin catabolism in erythrocytes which were being formed at the time of the glycine-2-$^{14}$C injection. The evidence that the early labeled $^{14}$CO and the CO production not explained by RBC hemoglobin catabolism may in part arise from catabolism of hepatic heme depends principally on studies of early labeled bile pigment (Robinson 32) plus the finding of equimolar CO and bilirubin during early labled CO and bilirubin production (Landaw et al 8, Engel et al 33). The rate of hepatic heme turnover has been related to two hepatic enzyme systems, both of which can be induced under various conditions. The MHO system which has been discussed above, in addition to being involved in all or most of the degradation of hemoglobin, appears to be a determinant of the rate of degradation of hepatic heme (Bakken et al 31). The activity of hepatic mixed function enzyme systems involved in oxidations of drugs, carcinogens, and other substances has also been related to the catabolic rate of hepatic heme (Remmer & Merker 34). It is well known that administration of drugs which are catabolized by this system results in very large increases in hepatic heme (Remmer & Merker 34). Induction of hepatic microsomal heme with phenobarbital has been a particularly valuable investigative tool in studying degradation of hepatic heme. Phenobarbital induces delta aminolevulinic synthetase (ALA-S), the rate-limiting enzyme for heme biosynthesis in the liver, and therefore causes an increase in hepatic heme synthesis, as well as causing an increase in microsomal cytochromes (Marver et al 35, Levitt et al 36). Landaw et al (8) found that phenobarbital was followed by an increase in $^{14}$CO and $^{14}$C-bilirubin in the early labeled peak after glycine-2-$^{14}$C injection in the bile cannula rat preparation. Administration of 100–300 mg phenobarbital/day to normal male subjects for seven days caused almost a doubling of $\dot{V}_{CO}$ (Coburn 37); total body heme measured by CO dilution also increased 5–10% after phenobarbital without a change in hemoglobin concentration in blood. Similar findings occurred following diphenylhydantoin. On the basis of these data it can be argued that the animal data are applicable to normal man, and since $\dot{V}_{CO}$ can be increased by drugs which induce increases in liver heme, that liver heme turnover probably contributes significantly to CO and bilirubin production in normal man. Presumably the increase in $\dot{V}_{CO}$ occurring with progesterone is due to a similar mechanism (Delivoria-Papadopoulous et al 21).

There is some evidence that induction of activity of the MHO system does not increase the activity of drug metabolism systems (Tenhunen et al 30). The effect of inducing drug metabolism enzymes on MHO activity is apparently unknown.

It is possible that variation in normal $\dot{V}_{CO}$ in the different series shown in Table 1 and daily and weekly variation in given subjects is related to variation in hepatic heme turnover rates. The rate of synthesis of ALA-S has been shown to have a reciprocal relationship to caloric uptake (Tschudy et al 38), suggesting that variation in $\dot{V}_{CO}$ may be related to diet. A low caloric diet period was followed by an increase in fecal excretion of protoporphyrin in a patient with erythropoietic protoporphyria (Redeker & Sterling 39). There is also evidence that patients with acute intermittent porphyria show rapid increases in urinary excretion of delta amino levulinic acid and porphobilinogen following caloric restriction (Welland et al 40). Recently Lundh et al (20) have found that caloric restriction causes a prompt increase in $\dot{V}_{CO}$ (up to 300%), but this finding is still controversial since a similar study showed only a small increase in $\dot{V}_{CO}$ following caloric restriction (Bloomer et al 41).

It might be suspected that measurement of CO production is not a precise method of quantitating RBC life span due to CO production arising from degradation of nonhemoglobin heme. Data available at present from patients with hemolytic anemia, however, suggest that the method may be useful in most patients due to the apparently relatively small fraction of $\dot{V}_{CO}$ contributed by nonhemoglobin heme degradation. Comparison of $\dot{V}_{CO}$ and RBC life span computed independently using labeling techniques have been performed in only two studies to our knowledge. In our study (Coburn et al 42), correlation between $\dot{V}_{CO}$ and RBC survival was excellent. All of the patients produced more CO than could be explained by circulating RBC destruction, but in only one patient was the amount of "unexplained" CO larger than that found in normal man. When it was later found that phenobarbital and other drugs which induce microsomal heme cause an increase in $\dot{V}_{CO}$, we checked the charts of all of the patients in this series and found that only this patient was taking a barbiturate sleeping preparation. Therefore, excluding data from this patient, the hemolytic anemia $\dot{V}_{CO}$ measurements support the concept that nonhemoglobin heme catabolism remains relatively small in these patients. This seems to be in conflict with the finding, discussed above, that the hepatic MHO activity in rats is induced with experimental hemolytic anemia (Tenhenen et al 30), if increases in activity of this system are associated with increases in rate of hepatic heme degradation to CO (Bakken et al 31).

A comparison of $\dot{V}_{CO}$ and RBC destruction was also performed by Logue et al (23) who found close correlation between CO and RBC survival. Due to uncertainties in their technique of estimating blood COHB from the $P_{CO}$ in the rebreathing system discussed above, little can be made at this time of their finding large quantities of CO not explained by hemoglobin catabolism in tagged RBCs.

It is now possible to measure bilirubin production precisely and it has been found that this measurement allows a precise determination of red cell life span in "most patients" with hemolytic anemia (Berk et al 43). These data should apply to the question of the value of the $\dot{V}_{CO}$ measurement in estimating RBC life span in these patients because of evidence for equimolar relationship of CO and bilirubin during heme catabolism (Engel et al 33, Landaw et al 8).

In patients with ineffective erythropoiesis (or ineffective reticulocytosis) circulating RBC life span cannot be estimated from $\dot{V}_{CO}$ measurements, but combinations of RBC labeling techniques and $\dot{V}_{CO}$ measurements are useful in quantitating ineffective erythropoiesis (White et al 44). It has been shown that $^{14}CO$ production occurs in bone marrow following glycine-2-$^{14}C$ (White 45).

Several authors consider that the $\dot{V}_{CO}$ measurement is a useful technique in assessing results of therapy in hemolytic anemia (Besinger et al 46, Logue et al 47). It seems worthwhile to continue to evaluate the usefulness of the $\dot{V}_{CO}$ measurement because of the advantages of this technique: (*a*) it does not require radioisotopes; (*b*) the measurement can be repeated every day if necessary; (*c*) it has a very high sensitivity, especially in patients with a high rate of CO production.

Abnormalities in RBC life span can also be studied by administering glycine-2-$^{14}C$ and measuring production of $^{14}CO$. The first such study was performed by White et al (45) who followed $^{14}CO$ production in a normal human subject for 160 days. Landaw et al (48) refined the technique and obtained beautiful data in rats and other experimental animals from which the rate of random hemolysis, mean potential and overall life span, spread of life spans around this mean, and the fraction of RBCs dying of senescence could be computed.

Measurement of the maximal rate at which hemoglobin can be catabolized to CO has been performed in anesthetized dogs (Coburn & Kane 49). Intravenous injections of large quantities of chemically damaged erythrocytes resulted in a prompt increase in $\dot{V}_{CO}$ to about 40 times the computed normal rate originating from RBC catabolism. The finding that maximal $\dot{V}_{CO}$ occurred as early as 30–60 min following sequestration of the injected cells may be in conflict with data obtained on rat liver which indicated that the activity of the MHO system was sufficient only for normal rates of heme catabolism, although activity could be induced severalfold by experimental hemolytic anemia (Tenhunen et al 30).

The rebreathing method for measurement of $\dot{V}_{CO}$ has been adapted to investigation of RBC catabolism under conditions where both extravascular and intravascular hemolysis are occurring (Wallace et al 17). Advantage was taken of the finding that catabolism of plasma hemoglobin, probably mostly haptoglobin-hemoglobin complex, occurs at such a low rate that the effects on $\dot{V}_{CO}$ are very small, whereas extravascular hemolysis results in prompt and large increases in $\dot{V}_{CO}$. Thus, extravascular hemolysis can be computed from increases in $\dot{V}_{CO}$, compared to a control value, and intravascular hemolysis from changes in plasma hemoglobin.

There are now considerable data available related to the stoichiometry of CO and bilirubin production and heme catabolism. Equimolar production of CO and bilirubin was found in the rat liver microsomal preparation as cited above (Tenhunen et al 29). Landaw et al (8) have demonstrated equimolar production in intact rats with bile cannulas following injections of $^{14}$C-hematin and also following glycine-2-$^{14}$C. Engel et al (33) obtained evidence for equimolar production of CO and bilirubin in normal man with simultaneous measurements of CO production and bilirubin production. It is possible, however, that measurements of CO production cannot always be equated to bilirubin production. Landaw et al (8) administered the porphyrogenic drug allylisopropylacetamide (AIA) to bile duct cannula rats and found that molar ratios of CO/bilirubin following $^{14}$C-hematin injections became greater than unity. A similar finding was also seen following glycine-2-$^{14}$C injections. AIA probably profoundly alters breakdown of heme in the liver, but these experiments did demonstrate that under the conditions of their studies there are alternative routes of degradation of heme to bilirubin which appear to occur after cleavage of the ferriprotoporphyrin ring. Schacter et al (50) obtained evidence of another alternate route of heme degradation. They stimulated NADPH-dependent lipid peroxidation by ADP and $FeCl_3$ in the rat liver which resulted in a loss of microsomal heme and cytochrome P-450. CO production was nearly equimolar to total heme degraded, suggesting cleavage of the ring at only one site per molecule, but less than molar yields of bilirubin were recovered. This mechanism of hepatic microsomal heme degradation appeared to be distinct from the NADPH-dependent MHO system. It is not known at present if AIA heme degradation and/or heme degraded with NADPH-dependent lipid peroxidation stimulation are exaggerations of normal alternate routes or completely abnormal pathways.

Most workers have found less than molar yields of bilirubin and CO during hemoglobin and hematin catabolism, suggesting there are alternate pathways not involving cleavage of the ferriprotoporphyrin ring. There is some evidence that increasing blood COHB to 10–12% may cause inhibition of the MHO system and stimulate alternate pathways of heme catabolism not involving CO production (Coburn et al 7).

The best evidence, in my opinion, that CO is not produced in significant quantities by mechanisms not involving heme catabolism is the finding of equimolar CO and bilirubin production in normal man (Engel et al 33). It is known that CO is produced by some bacteria and other metabolic processes, i.e. oxidation of phenols, but apparently this is not significant in vivo. It has been stated that ingested hemoproteins can contribute to CO production (Engel & Levitt 51). Exposure to dichloromethane results in an increase in blood COHB which appears to be due to catabolism of this compound, rather than stimulation of heme catabolism (Stewart et al 52).

The question whether nonhepatic nonhemoglobin hemoprotein degradation results in significant CO is also not answered. It seems less likely, now, that myoglobin catabolism results in CO production since this compound is not a successful substrate of the MHO system (Tenhunen et al 28).

## SUMMARY

It has been proposed that oxidation of CO may be the basis of energy production in some extraterrestrial civilization (Weinstock & Niki 53). Certainly endogenous CO metabolism is of less importance to life on earth; however, measurements and studies of CO physiology have led to a better understanding of heme catabolism and are likely to continue to be useful in the future.

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# HUMAN PROLACTIN

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Until the recent isolation of human prolactin (1, 2) considerable doubt existed whether the human pituitary secretes a separate prolactin molecule (3). Because of the difficulties encountered in separating human growth hormone (hGH) from human prolactin (hPRL) and also because the primary structure of hGH resembles that of sheep prolactin it was suggested that in man, lactation and growth are controlled by a single protein (4). However, in almost all other species with the exception of primates (man and monkey) it was relatively easy to purify growth hormone and prolactin as separate hormones using standard bioassays for their identification. The classical assay for growth hormone depends upon an increase in the body weight of hypophysectomized rats or a widening of the tibial epiphyseal cartilage width, while prolactin is detected by its ability to stimulate the growth of the pigeon crop sac mucosa or to cause lactational changes in the rabbit mammary gland. Prolactin and growth hormone from all species had either activity but not both. In the case of human and monkey pituitary extracts, the growth hormone or prolactin preparations invariably produced positive responses in both assays although the ratio of growth hormone to prolactin varied considerably among the different preparations. Thus it was understandable that the failure to isolate human prolactin led some investigators to conclude that hGH has intrinsic PRL activity and that possibly PRL does not exist as a separate entity in man.

A number of clinical and experimental observations, however, suggested that a separate human PRL molecule must be present in man as in other species. Acromegalic patients with elevated hGH concentrations only occasionally develop galactorrhea, whereas most subjects with galactorrhea or women who are breast feeding have normal serum hGH values. Indeed when plasma extracts were prepared from patients with galactorrhea, it was found that despite low or undetectable serum hGH values measured by radioimmunoassay, serum prolactin concentrations determined by bioassay were elevated (5). Furthermore, some patients with a hereditary form of isolated growth hormone deficiency (ateliotic dwarfism) lactate normally despite the absence of hGH (6). Stalk section of the pituitary which does not produce elevated serum hGH concentrations leads to galactorrhea in some subjects. Moreover, an extract of a pituitary tumor from a patient with galactorrhea was rich in prolactin but deficient in growth hormone (7). Preincubation of the extract with antiserum to hGH did not neutralize the prolactin activity, demonstrating that hPRL and hGH are immunologically different. A similar observation was made by Frantz & Kleinberg (8) who noted that the prolactin activity in the plasma of postpartum lactating women and patients with galactorrhea could not be neutralized by anti-hGH serum whereas the prolactin activity in sera from patients with acromegaly was neutralized. These clinical and experimental observations were incompatible with the suggestion that hGH and hPRL are one and the same hormone in man.

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Histological and subsequently immunological studies also supported the notion of a separate hPRL molecule. Erdheim & Stumme (9) first documented the extensive histological changes that are seen in the pituitary during pregnancy and lactation. They identified a distinctive cell type "the pregnancy cell" which Purves (10) suggested is the cell which secretes prolactin in man. In routine pituitary glands obtained at postmortem this cell type could not be identified without the use of special stains which were developed by Herlant & Pasteels (11) and later by Goluboff & Ezrin (12). With these special techniques human prolactin cells were identified in tissue culture of pituitary glands obtained from women who died during pregnancy, as well as in prolactin-secreting pituitary tumors. Goluboff & Ezrin showed that the prolactin cell is an acidophil which stains bright red with carmoisine. During pregnancy or in the postpartum period prolactin cells constituted upwards of 50% of the acidophil population. Immunofluorescence studies showed that antisera to hGH or human placental lactogen (hPL) did not localize in the prolactin cell (13) suggesting that hGH or hPL and hPRL must be immunologically different. Subsequently, Herbert & Hayashida made the very important and critical observation (14) that antiserum to sheep prolactin localized in these cells, providing the first direct histological evidence that monkey, human, and sheep prolactin are immunologically related. This finding immediately provided investigators with an immunological tool for identifying human and monkey prolactin. Pasteels confirmed the localization of antisheep prolactin in pregnancy cells (15) and Frantz (16) reported that in sera from patients with galactorrhea, the prolactin activity that was not neutralized by anti-hGH serum was completely inactivated by antiserum to sheep prolactin.

In studies in our laboratory we obtained direct evidence for the synthesis of human and monkey prolactin by incubating human (17) and monkey (18) pituitary tissue fragments in the presence of $^3$H-labeled leucine. When the proteins in the incubation media were fractionated by gel filtration, a single large peak of radioactive protein was identified as primate prolactin because of its cross-reaction with antiserum to sheep prolactin but not with antiserum to hPL or hGH (19). Moreover tumor fragments from a patient with galactorrhea synthesized prolactin almost exclusively as judged by immunoprecipitation studies with anti-ovine prolactin (20, 21). These findings indicated that the common antigenic determinants which were shared by human and sheep prolactin were absent in hGH and hPL. We took advantage of this immunological difference by coupling specific antibodies to hPL to Sepharose and used this immunoadsorbent to remove all the GH from human and monkey pituitary fractions without the loss of all prolactin activity (22). This procedure provided the first reliable and reproducible method of separating human and monkey prolactin from growth hormone. These prolactin-rich, growth hormone-free preparations were used to immunize rabbits. The antisera which were obtained cross-reacted with sheep prolactin and were used to develop a specific radioimmunoassay for human and monkey prolactin (23). Although the assay was not very sensitive it enabled us to identify human prolactin specifically without interference by hGH. This radioimmunoassay subsequently was modified (24) and proved to be invaluable in the purification of human prolactin (2) as many fractions could be analyzed for prolactin easily and rapidly.

## THE PROLACTIN CELL

The prolactin cell has been identified in human and monkey pituitary glands by light microscopy using special staining techniques and immunofluorescence localization methods. Herlant & Pasteels (11) cite three different staining procedures which are useful in identifying prolactin cells: Herlant's tetrachrome stain, the methasol blue-PAS-orange-G stain, and Brooke's stain. The number of prolactin cells in the pituitary varies with different physiological states. In the infant monkey erythrosinophilic prolactin cells are virtually absent among the anterior pituitary cells. In adult male and nonlactating female monkeys the frequency of prolactin cells ranges between 2.5–11.1%. In man Goluboff & Ezrin (12) also found that the prolactin cells are virtually absent in most specimens examined with several exceptions. In pituitaries from women who died during pregnancy or in the postpartum period, or in some male patients treated with estrogens, there is a great abundance of prolactin cells. Antibodies to ovine prolactin coupled to fluorescein isothiocyanate localize in "pregnancy cells," whereas antibodies to hGH do not. These experiments confirm a similar observation in monkeys and show clearly that hPRL and hGH are immunologically different and that each is secreted by a different cell type in primates as well as in other mammals. Prolactin cells are also recognizable by electron microscopy (25). They are distinguished by a lamellar concentric pattern of rough endoplasmic reticulum and the secretory granules containing prolactin are somewhat larger than the somatotropin granules. An even more reliable discriminating feature is the polymorphism of the prolactin granules. Within the same cell, elongated, triangular, oval, and irregular shaped granules of various sizes are seen. This appearance contrasts sharply with the more uniform size of the smaller somatotropin granules. Microtubules are often prominent in prolactin cells suggesting that the secretory process is especially active in this cell type.

## THE PURIFICATION AND CHEMISTRY OF HUMAN PROLACTIN

Several explanations might be offered for the previous failure to purify hPRL. One of the principal reasons is that hGH constitutes 5–10% of the dry weight of pituitary powder while the hPRL content is around 0.1%, that is, a 50- to 100-fold greater amount of hGH. The second reason is that hGH, unlike growth hormone from other species, has 10–20% of the prolactin activity of the best sheep prolactin

**TABLE 1. Amino Acid Sequence of $NH_2$ Terminal Residues of Human Placental Lactogen, Growth Hormone, Human Prolactin, and Sheep Prolactin[a]**

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hPL | $NH_2$ | Val | Gln | Thr | Val | Pro | Leu | Ser | Arg | Leu | Phe | Asp | His | Ala | Met | Leu | Gln | Ala | His | Arg | Ala | His | Gln | Leu |
| hGH | $NH_2$ | Phe | Pro | Thr | Ile | Pro | Leu | Ser | Arg | Leu | Phe | Asp | Asn | Ala | Met | Leu | Arg | Ala | His | Arg | Leu | His | Gln | Leu |
| hPRL | $NH_2$ | Leu | Pro | Ile | Cys | Pro | Gly | Gly | Ala | Ala | Arg | Cys | Gln | Val | Thr | Leu | Arg | Asp | Leu | Phe | Asp | Arg | Ala | Val |
| oPRL | $NH_2$ | Thr | Pro | Val | Cys | Pro | Asn | Gly | Pro | Gly | Asn | Cys | Gln | Val | Ser | Leu | Arg | Asp | Leu | Phe | Asp | Arg | Ala | Val |
| | | | | | | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | |

[a] Data obtained by H. Niall, Massachusetts General Hospital, Boston, Massachusetts.

standards. Therefore, when total prolactin activity is monitored during chromatography procedures by bioassay, one invariably follows the fractionation of hGH. This can readily be appreciated by the following example. If the potency of pure hPRL is 30 IU/mg and that of hGH is 5 IU/mg, then 90% of the total prolactin activity in pituitary homogenates is due to hGH because of the 50-to 100-fold greater amount of hGH in the pituitary. During pregnancy the number of prolactin cells and the prolactin content of the pituitary increase dramatically while the number of hGH cells decrease. Lewis et al (26) were able to identify a "new" electrophoretic band with a slightly different mobility from hGH in the homogenate of a pituitary gland obtained from a woman who died during pregnancy. They correctly identified this component as hPRL and developed a method for its purification based on the distinctive electrophoretic mobility of this new component. (1)

In our studies on the purification of hPRL we utilized the specific radioimmunoassay for hPRL. The starting material for the isolation was the supernatant which remains after precipitating hGH with 50% ethanol at pH 8.5 in the Raben procedure for purifying hGH (2). The purified hPRL preparation appeared homogeneous and identical with the Lewis hPRL preparation by several criteria. Subsequently we developed a method for the large scale purification of hPRL from pituitary glands which were frozen at autopsy (27). The yield of hPRL was 40–50 μg/pituitary compared with 10 μg obtained by Lewis. The biological potency of the preparation was 30 IU/mg in a mouse mammary gland assay and it contained less than 0.5% hGH as measured by radioimmunoassay. The N-terminal amino acid was leucine and not phenylalanine which is found in hGH. Table 1 shows a comparison of the amino acid sequence of the first 24 N-terminal residues of human and sheep prolactin, hGH, and hPL (28). There is extensive homology between sheep prolactin and hPRL and between hGH and hPL but not between hGH and hPRL. Lewis et al (29) reported that 75% of the tryptic peptide maps of hPRL and sheep prolactin are identical. It is simply a matter of time until the entire amino acid sequence of hPRL is completed, but the available evidence leaves no room to doubt the existence of a separate prolactin molecule in the human pituitary gland. With the knowledge of the sequence of hPRL, structure-function studies will be possible to determine which residues are responsible for the biological activity of hPRL.

## ASSAYS FOR PROLACTIN: BIOASSAYS, RADIORECEPTOR ASSAYS, AND RADIOIMMUNOASSAYS

The more traditional bioassays for prolactin—the pigeon crop sac assay (30) and the rabbit intraductal mammary gland assay (31)—are cumbersome, but more importantly they lack sufficient sensitivity to measure circulating levels of prolactin. Moreover, hGH is as potent as PRL in the rabbit assay and also gives positive responses in the pigeon assay (31). To try to overcome these drawbacks a number of investigators have developed bioassays which can detect normal and especially elevated serum prolactin levels in unextracted plasma samples. These assays depend on prolactin inducing histological changes in midpregnant mouse mammary glands (32), or in pseudopregnant rabbit mammary gland (33) maintained in tissue cultures for 3–5 days. Other bioassays have utilized the stimulatory effect of prolactin on the induction of $^{32}$P-casein synthesis (34) or the induction of *N*-acetyl lactosamine synthetase (the galactosyl transferase of lactose synthesis) in midpregnant mouse breast tissue (35). In all of these assays both hPL and hGH give positive responses. This is a serious problem in measuring serum prolactin during pregnancy or in cases of acromegaly. In order to make the assay specific both of these hormones must be neutralized with specific anti-hPL or anti-hGH serum. Alternatively, the hGH concentration in any sample can be measured by a specific radioimmunoassay for hGH and the total prolactin activity can be assumed to be that due to hPRL plus hGH.

Several groups have developed radioimmunoassays for hPRL. Bryant et al (36) developed a radioimmunoassay using antiserum to an "hPRL preparation" obtained from fetal pituitaries in tissue culture. A mixed heterologous assay using antiserum to sheep prolactin and porcine prolactin tracer was reported by Jacobs et al (37), while Midgley (38) and L'Hermite et al (39) were able to measure serum prolactin using a homologous radioimmunoassay for sheep prolactin. In our own laboratory we initially developed a radioimmunoassay using rhesus monkey prolactin tracer and antiserum to hPRL. However, with the availability of a purified hPRL standard we have substituted this material for our tracer (40).

A radioreceptor assay utilizing membrane receptors derived from midpregnant mouse mammary tissue has been reported (41). Subsequent reports have suggested that a large amount of nonspecific binding of damaged prolactin to the tissue presents a serious problem (42). In our laboratory we have used midpregnant rabbit mammary glands obtained from animals which were given cortisone acetate and hPL for 4 days (43). With this technique we have been able to detect 2 ng/ml of hPRL in a 1 hr assay. Other lactogenic hormones which inhibit the binding of hPRL-$^{125}$I to the tissue receptors include hGH and hPL, as well as prolactin, but not growth hormone from other species. The great advantage of this method is that assay results can be obtained rapidly. The homogenate of rabbit mammary glands is stable when frozen and large amounts of mammary tissue can be obtained from a single animal. Undoubtedly this form of assay will replace other types of bioassays for prolactin to a large extent.

When comparing bioassays, tissue receptor assays, and radioimmunoassays, several important factors require consideration: specificity, sensitivity, precision, reproducibility, time, cost, and ease with which assays can be performed. It is apparent that the bioassays are not specific for hPRL but measure several lactogenic substances including hGH. The sensitivity of the radioimmunoassays and radioreceptor assays are very similar and significantly greater than that of most bioassays. The precision and reproducibility of radioimmunoassays are generally considerably better than that of bioassays. This is evident when comparing dose response curves by the two methods. In Turkington's assay (34), for example, an increase of 500 cpm/mg $^{32}P$ incorporation above the control encompasses prolactin concentrations from 5–500 ng/ml. The lactose synthetase method yields very similar dose-response curves. The chief advantage of radioimmunoassays, however, is the ease and facility with which large numbers of samples can be assayed, and the major drawback of the method is that immunoreactivity of a hormone may not correlate with its biological activity. For example, prolactin may be inactivated in serum and any biologically inert breakdown products which are formed would still cross-react in the assay provided they contain the antigenic determinants. Fortunately in the case of hPRL Frantz et al (16) found a very high degree of correlation between the results obtained by bioassay and immunoassay in literally hundreds of samples obtained from a great variety of patients. Hence, the theoretical consideration outlined above does not constitute a serious deterrent to the use of radioimmunoassays for measuring serum prolactin in man.

Since virtually all serum samples including those from patients with idiopathic hypopituitarism contain prolactin as measured by radioimmunoassay, it is obvious that in those bioassays in which the control incubation media contains 10–50% male serum it will not be possible to determine normal serum prolactin concentrations accurately. Hence the suggestion that normal serum prolactin values are less than 2.5 ng/ml must be viewed with some scepticism. However, estimates of elevated prolactin levels by bioassay and radioimmunoassay in at least one extensive comparison (16) are surprisingly similar (correlation coefficient 0.90). This study is especially valuable because serum prolactin concentrations in a great variety of clinical conditions were compared by the two methods.

## SERUM PROLACTIN CONCENTRATIONS IN DIFFERENT CLINICAL STATES

The serum prolactin levels which have been found in various clinical circumstances will be reviewed. The actual values obtained may require slight modification in some cases, as initially no common hPRL standard was available to all investigators; recently the Medical Research Council of Great Britain has issued an interim standard. Serum prolactin concentrations can be detected in most subjects throughout life; Table 2 lists representative values obtained in our laboratory. Our findings (24, 40) are in close agreement with the results reported by Jacobs et al (37) as well as those found by Frantz et al (16).

In the full term newborn, prolactin levels are grossly elevated compared with adults, and the hormone concentration remains elevated for a longer period in premature infants (44). Adult levels are reached somewhere between 3–6 months of age.

At puberty in female but not male subjects serum prolactin levels appear to increase but only very modestly compared to the rise in LH and FSH that occurs at this time. There is, however, considerable overlap in values between the two sexes. We presume that the increase in estrogen secretion at puberty leads to an increase in prolactin because some investigators have found that estrogen infusions cause a prompt increase in serum prolactin (39). Other investigators have reported that when an amount of estrogen which will decrease FSH (follicle-stimulating hormone) and LH (luteinizing hormone) levels is given, prolactin levels did not change (38). In our experience, the chronic administration of large

TABLE 2. Serum prolactin concentrations in different clinical circumstances

| | Mean | Range |
|---|---|---|
| Children | 10.8 | 7–17 |
| Adults | | |
| male | 7 | 0–28 |
| female, follicular phase | 10 | 4–20 |
| female, luteal phase | 11 | 5–42 |
| Pregnancy | | |
| 1st trimester | 30 | 7–70 |
| 2nd trimester | 60 | 20–450 |
| 3rd trimester | 120 | 36–600 |
| term | 200 | 50–600 |
| Amniotic Fluid | | |
| <20 weeks | 2130 | 1700–50 |
| 20–39 weeks | 1600 | 500–5800 |
| term | 1120 | 350–1500 |
| Postpartum | | |
| day (1) | 150 | 50–300 |
| day (7) | 40 | 7–60 |
| Nursing | | |
| 1st week, pre | 100 | 80–230 |
| 1st week, post (30 min) | 140 | 90–300 |
| 3rd week and later, pre | 10 | 5–30 |
| 3rd week, post (30 min) | 250 | 160–400 |
| 3rd week, post (60 min) | 120 | 80–150 |
| Newborn | | |
| cord blood | 200 | 100–500 |
| 1 week | 75 | 20–150 |

amounts of estrogen to male patients with prostatic cancer only occasionally produces elevated prolactin values. Our initial studies in 10 women suggested no change in serum prolactin during the menstrual cycle although there was considerable daily variation in serum prolactin in different women. Upon reexamination there is some evidence that prolactin concentrations are slightly higher in the luteal phase (44) and that the variance, that is, the amplitude of the daily variations in serum prolactin, is also greater in the second half of the cycle. Additional studies will be required to confirm these findings. However, it would be surprising if prolactin has any effect on the corpus luteum in women in view of Midgley's (38) findings that radioautographs failed to show any binding of hPRL-$^{125}$I to ovarian tissue, whereas $^{125}$I-HCG (human chorionic gonadotropin) binding was readily demonstrable. Conversely, hPRL-I$^{125}$ was bound to mammary tissue whereas $^{125}$I-HCG was not.

Insufficient data are available to know whether serum prolactin values in post-menopausal women decrease to male levels. Serum prolactin concentrations show a diurnal variation, the highest values occurring 2–3 hr after the onset of sleep, and remain elevated much longer than the serum hGH levels (45, 46). Serum prolactin levels may still be somewhat above the presleep concentration in the early morning hours.

During pregnancy there is a progressive increase in serum prolactin concentrations reaching maximal values at term (24). Postpartum the concentrations fall quite rapidly and return to normal by 3 weeks in mothers who do not breast feed. There is no sudden surge of prolactin levels on day 2–4, when breast engorgement usually occurs. In the first week postpartum in those women who nurse, serum prolactin levels which are still elevated show a definite but small increase 30 min after the onset of nursing (47). From the 2nd to 3rd week until 2–3 months postpartum, basal prolactin concentrations are about twice those found in nonpregnant females. In this period, breast feeding frequently but not uniformly causes a dramatic increase in prolactin concentration, values increasing 10- to 20-fold within 15–30 min. Finally, beyond the 3rd month postpartum basal prolactin values are similar to those found in the nonpregnant state and nursing produces no increase as it did in phase II. In one patient with isolated growth hormone deficiency (ateliotic dwarf), serum hPRL showed a similar increase during pregnancy and postpartum as in normal individuals (47).

In the few samples which have been examined, fetal prolactin concentrations have ranged from 2–200 ng/ml. Usually, corresponding maternal hPRL concentrations have been higher. One of the completely unexpected findings was the extremely high prolactin concentrations in amniotic fluid (40). In the first trimester concentrations as great as 10,000 ng/ml have been found compared to maternal or fetal serum values of 20–50 ng/ml. At term amniotic fluid prolactin concentrations have usually declined to the 1000 ng/ml range (300–1500 ng/ml). The amniotic fluid prolactin immunologically, electrophoretically, and chromatographically appears identical with pituitary prolactin. However, definitive chemical evidence of the identity of the two has not been reported. The source of the amniotic fluid prolactin is uncertain.

Several additional physiological stimuli will cause an increase in hPRL, e.g. exercise (48) and psychological or postsurgical stress (20, 40). Most patients undergoing surgical procedures under general anesthesia will show a considerable increase in serum hPRL for 12–24 hr. Generally female patients exhibit greater increases than do male patients.

Several reports indicate that the administration of prolactin to rats (49) and man (50) causes an increase in sodium and water retention. However, we found no systematic change in serum hPRL when subjects were on a high (100 g) vs a low (10 g) sodium diet. Moreover, after the administration of furosemide, subjects who lost 3% of their body weight showed no change in serum hPRL. Finally, patients with prolactin-secreting pituitary tumors and with serum hPRL values greater than 10,000 ng/ml had no evidence of sodium retention.

## THE CONTROL OF PROLACTIN SECRETION: EFFECTS OF DRUGS

A large number of pharmacological agents may affect serum hPRL concentrations. To understand the mechanism of action of these drugs a brief review of the control of prolactin secretion is appropriate. Most of the concepts regarding the control of prolactin have been developed from experimental work done principally in rats (51). We assume that the regulation of prolactin secretion in man is very similar. The secretion of prolactin, like that of melanocyte-stimulating hormone, but unlike the other pituitary hormones, is inhibited by a hypothalamic substance termed prolactin-inhibiting factor (PIF). There is also more limited evidence for a prolactin-releasing factor (PRF). These substances by analogy with the other hypothalamic releasing factors are synthesized in the hypothalamus, stored in the median eminence, and transported to the anterior lobe in the portal venous system which passes down the pituitary stalk. There is some evidence that PIF is synthesized in the same region of the hypothalamus as luteinizing- and follicle-stimulating hormone releasing factor (LHRF) which would account for the common association of galactorrhea and amenorrhea. The secretion of PIF is controlled by catecholamines via dopaminergic fibers. Thus an increase of catecholamines as a result of the administration of L-dopa causes an increase in PIF secretion which inhibits prolactin secretion by the pituitary in man (52, 53). A second group of agents which inhibit prolactin secretion are ergot drugs. These agents act directly on the pituitary prolactin cells to inhibit prolactin secretion (54), but recent evidence has shown that the agents also produce a marked change in catecholamine levels in the hypothalamus (55). Hence, the drugs may have a combined action on the pituitary as well as on the hypothalamus.

Two agents which cause an increase in prolactin secretion are psychotropic agents (especially phenothiazines) and thyrotropin-releasing hormone (TRH), acting at the level of the hypothalamus and pituitary gland respectively. One phenothiazine, chlorpromazine, has been investigated most extensively (52, 56, 57). This agent depletes the effective catecholamine levels in the hypothalamus, producing a decrease of PIF which leads to an increase of prolactin secretion.

TRH, which was once considered to be the specific hypothalamic factor controlling TSH (thyroid-stimulating hormone) secretion, turns out to be a very potent releaser of prolactin in a number of species. TRH, L-dopa, and chlorpromazine have been employed in the functional evaluation of pituitary prolactin secretion in man (52).

A second regulatory system for controlling prolactin secretion has been proposed because infusions of L-tryptophan but not other amino acids caused a marked increase in prolactin secretion without increasing TSH levels (58). Prior treatment with methysergide maleate blocked these effects of L-tryptophan suggesting that they depend upon conversion of tryptophan to serotonin. On the basis of these studies it was suggested that hypothalamic regulation of prolactin secretion in man is mediated by a balance between inhibitory catecholaminergic and stimulatory serotoninergic mechanisms. This study needs to be confirmed because, unlike these investigators, we found that approximately 50% of patients receiving arginine infusions also had a significant increase of serum prolactin (21).

L-Dopa and ergocryptine have been used therapeutically to decrease prolactin secretion (59, 60). With the help of pharmacologic agents one can distinguish three patterns of prolactin secretion in patients with hypopituitarism (61). In one group of patients with panhypopituitarism basal prolactin levels were generally less than 3.0 ng/ml and failed to increase or decrease with TRH, chlorpromazine, or L-dopa respectively. Tests of all other pituitary functions were abnormal as well. A second group of patients with extensive hypothalamic disease as the basis of their pituitary dysfunction showed a slight elevation in basal prolactin values, a definite response to TRH, no increase after chlorpromazine, and a subnormal decrease of serum prolactin levels after L-dopa. Finally a third group of patients had definite but subnormal responses to all 3 agents. It is important to utilize all 3 tests in order to avoid the erroneous conclusion that any patient has a deficiency of prolactin secretion simply because he fails to exhibit an increase following chlorpromazine (57).

Patients who receive L-dopa for Parkinson's disease generally have undetectable levels of prolactin whereas psychiatric patients receiving large doses of phenothiazines frequently but not invariably have elevated serum prolactin levels. Women on tranquillizers more commonly have elevated levels and their values are generally higher than those of male subjects (40). The increase in prolactin accounts for the frequency of galactorrhea in these patients. Both L-dopa and ergocryptine have been used in the treatment of patients with elevated prolactin levels secondary to pituitary or hypothalamic disease (58–60). Both agents can inhibit prolactin secretion in patients with pituitary tumors and galactorrhea, but unfortunately when therapy is discontinued prolactin levels usually return to pretreatment levels (58) with a recurrence of the galactorrhea. An interesting accompaniment of the cessation of galactorrhea is the return of normal menses in some patients. This association suggests that the two symptoms arise from a common or closely linked hypothalamic disorder. Pilot studies with L-dopa and ergocryptine also have been initiated in the therapy of breast cancer and prostatic cancer (see below). A single dose of ergocryptine produces a more sustained suppression of prolactin levels (12–24 hr) than the 2–3 hr decrease found with L-dopa (60).

The increase in serum prolactin after TRH administration to human subjects was most surprising (62, 63). The initial reports have been amply confirmed and extended to other species including the monkey (64), cow, and pig (65). Indeed the rat appears to be one of the species least responsive to the effect of TRH on prolactin release. TRH added to pituitary fragments in vitro will directly stimuate PRL secretion, but the minimal effective dose seems large by comparison to the minimum effective dose of 5–10 μg TRH which will increase both TSH and PRL levels in man. In our studies we have found that the minimal dose which stimulates TSH release invariably increases PRL secretion. Moreover, the effective dose range for releasing TSH and PRL is the same. TRH produces a greater release of both TSH and PRL in women than men. Hypothyroid subjects release more and hyperthyroid patients release less PRL than normal subjects. This difference is the same as that found for TSH. The administration of thyroxine or $T_3$ to euthyroid subjects more easily inhibits the TRH- induced TSH than PRL release (66). The fact that most patients with hypothyroidism do not have elevated PRL levels under basal conditions and yet respond with an exaggerated increase in PRL and TSH to an even smaller dose of TRH than normal subjects do suggests to us that the increase of serum TSH in hypothyroidism is due to an altered sensitivity of the TSH-prolactin secreting cell to TRH. A large number of TRH analogues have been tested for prolactin-releasing activity in monkeys (64). Only those analogues which increased TSH secretion caused an increase in PRL secretion. In monkeys 1 μg TRH intravenously caused a 6-fold increase in PRL and only a 2-fold increase in TSH. In cows TSH increase was modest whereas PRL levels increased 5- to 10-fold after 100 μg TRH (65). In some patients with pituitary prolactin-secreting tumors TRH is able to increase serum prolactin levels even further. Occasionally TRH will also increase hGH concentrations in acromegalic subjects but this is an inconsistent effect. The PRL increase after TRH is large (>40 ng/ml) when one considers that the PRL and TSH content of the pituitary gland is 500 μg and 25 μg respectively. If one assumes that the volume of distribution of both hormones is the same, one can estimate that the complete prolactin store and more is released, whereas only 25% of the TSH store would have to be secreted to increase the PRL and TSH concentrations to the levels observed. Hence if a pituitary depletion assay were used to determine the potency of TRH in man, the tripeptide would be regarded primarily as prolactin-releasing hormone. TRH and PIF appear to compete for the same binding sites on the prolactin cell. In a patient who receives L-dopa 2 hr before TRH the effect of TRH on PRL release is completely inhibited whereas the TSH increase is not affected (67). The competitive inhibition is observed only transiently because if L-dopa is given 3–4 hr before the administration of TRH, no inhibition of PRL release is observed. L-Dopa pretreatment also blocks the chlorpromazine-induced prolactin release.

The effect of TRH on prolactin release is exaggerated in the postpartum period when increases in serum PRL may reach 120–200 ng/ml. (68) The greater increase probably reflects the larger store of prolactin in the pituitary. When TRH was given to patients who had stopped nursing for several days the increase in PRL levels was accompanied by marked breast engorgement and lactorrhea. The administration of TRH also increased both the fat and protein content of breast milk (69). The usefulness of TRH in augmenting milk yield and composition in women who have inadequate breast milk is being explored. Similar studies of the effect of TRH upon milk yield and composition in cows are underway. An orally effective galactogogue would be especially welcome in some underdeveloped countries where unsatisfactory hygienic standards in bottle-fed babies leads to a very high infant mortality rate.

The mechanism by which TRH acts on the lactotrope to increase PRL release is not clear. Tashjian has shown that the addition of TRH to cloned rat pituitary prolactin cells in tissue culture rapidly leads to a morphological change (70). Labeled TRH binds to the prolactin cell membrane and can be displaced for a period of several hours by the addition of unlabeled TRH, but thereafter the labeled TRH no longer can be dissociated from the cell.

It is still uncertain whether PRL release after TRH occurs because TRH is chemically related to another hypothalamic prolactin-releasing factor. Until the latter is isolated and chemically defined any such consideration must remain speculative. Prolactin secretion, therefore, appears to be under the control of PIF, TRH and "PRH." Whether the latter two are identical is unclear. It is certain, however, that TSH and PRL can be secreted independently. During nursing there is a large increase in PRL without any increase in TSH (authors' unpublished observations). Similarly PRL increases at night without any increase in TSH and finally during the stress of surgery serum PRL increases without any increase in TSH (authors' unpublished observations).

*Prolactin-secreting pituitary tumors.*—Prolactin-secreting pituitary tumors are probably the most common of all pituitary tumors (40). As most tumors do not produce galactorrhea or gynecomastia they have frequently been classified as "nonfunctional" chromophobe adenomas. Of 15 consecutive patients with pituitary tumors, 5 proved to have prolactin levels greater than 200 ng/ml. Serum prolactin values which range between 30–200 ng/ml are not diagnostic of a prolactin-secreting tumor because tumor extension to the hypothalamus or other diseases involving the hypothalamus may lead to a deficiency of PIF secretion causing modest elevations of serum prolactin. These diseases include infiltrative disorders such as sarcoidosis, histiocytosis X, metastases, etc. Considerable variation in prolactin levels occurs throughout the day, but in patients with prolactin-secreting tumors the lowest values rarely fall within the normal range. Some of the patients have astronomical levels of serum prolactin—concentrations greater than 500 ng/ml are not uncommon and in the occasional patient values may reach 15,000 ng/ml. As a group these values are clearly in excess of the hGH concentrations one sees in acromegalic subjects in whom values above 500 ng/ml are most unusual. Curiously, some of the patients with the highest levels have not had galactorrhea, or indeed any significant clinical abnormality which might suggest the presence of a prolactin-secreting pituitary tumor. This apparent anomaly emphasizes the fact that other hormones such as estrogens and corticoids are essential for the production of galactorrhea. Patients with a craniopharyngioma or indeed any infiltrative or destructive lesion of the hypothalamus may also have modestly elevated serum prolactin levels—values ranging from normal to 200 ng/ml. Another group of patients with serum prolactin in this range are patients with microadenomas of the pituitary gland. Dr. Jules Hardy of the University of Montreal (71) has demonstrated that these small adenomas can be selectively shelled out from the surrounding normal pituitary tissue using the transsphenoidal route. Two such patients whom we studied had galactorrhea and amenorrhea and serum prolactin values ranging between 70–90 ng/ml. When incubated in vitro the adenoma produced prolactin almost exclusively (20). Within 2 months of removal of one such tumor normal menses resumed and the patient became pregnant. The fascinating question raised by these findings is the relationship between the high prolactin levels and the amenorrhea. It is hard to imagine that the small adenomas weighing 20–40 mg produce gonadotropin deficiency by pressure. It seems possible that the elevated prolactin levels inhibit the normal cyclical discharge of LH and FSH either by an effect on the ovary or more likely by a direct action on the hypothalamus, or both. Similarly, resumption of normal menses and cessation of galactorrhea have been observed in patients whose elevated prolactin levels were lowered by L-dopa or ergocryptine (58, 60).

Prolactin-secreting tumors appear not to be completely autonomous. Serum prolactin levels are usually lowered with L-dopa and ergocryptine and raised with TRH but chlorpromazine is often without effect. After surgical removal of substantial amounts of the tumor the serum prolactin levels decline immediately, but often to a lesser degree than would be anticipated from the amount of tissue removed. Moreover, the levels then begin to increase again reaching values which approximate the concentrations found preoperatively. The rapid return of the elevated prolactin levels and the appropriate response to pharmacologic agents suggest that the tumor is not autonomous, but is stimulated by an excess of a hypothalamic-releasing factor. These observations indicate that the primary abnormality in at least some of these tumors is localized in the hypothalamus, a situation which also appears to be true for some patients with acromegaly (72). Most patients with acromegaly have normal serum prolactin levels. Similarly most patients with or without galactorrhea and prolactin-secreting tumors have normal serum hGH concentrations.

*Other disorders leading to increased prolactin secretion.*—Other conditions which may lead to elevated prolactin levels are summarized in Table 3. In our own studies we found that 20% of patients with advanced renal failure have elevated prolactin concentrations (73). There is only a poor correlation between the serum creatinine and prolactin level. One of the difficulties encountered in examining the pathogenesis of the elevation is that most of the patients are taking a variety of medications including antihypertensives such as methyldopa which may itself

TABLE 3. CAUSES OF ELEVATED PROLACTIN CONCENTRATIONS[a]

| Physiological | Pathological | Pharmacological |
|---|---|---|
| 1. Pregnancy | 1. Prolactin-secreting pituitary tumors<br>a. With galactorrhea<br>b. Without galactorrhea | 1. Thyrotropin-releasing hormone[b] |
| 2. Postpartum<br>a. Non-nursing mothers day 1–7<br>b. Nursing mothers after suckling | 2. Hypothalamic disorders leading to a decrease in the secretion of prolactin-inhibiting factor<br>a. Functional, e.g. Chiari-Frommel<br>b. Organic, e.g. craniopharyngioma, metastases, sarcoidosis | 2. Psychotropic drugs<br>a. Phenothiazine<br>b. Reserpine |
| 3. Newborn | 3. Pituitary stalk section | 3. Oral contraceptives after withdrawal |
| 4. Stress<br>a. Surgical<br>b. Psychological | 4. Hypothyroidism | 4. Estrogen therapy |
| 5. Exercise | 5. Nelson's syndrome | |
| 6. Nipple stimulation | 6. Renal failure | |
| | 7. Ectopic production by malignant tumors | |

[a] Galactorrhea and/or elevated prolactin have been reported or observed in some patients in each of the categories listed. However, some patients may develop galactorrhea with only a modest elevation of prolactin levels whereas other patients with great elevations of serum prolactin concentrations do not have any abnormal breast secretion. Because many conditions are associated with an increase in serum prolactin, the result of a single determination must be interpreted cautiously.

[b] As indicated in the text TRH or a chemically related substance may be a physiological regulator of prolactin secretion.

increase serum prolactin levels. Hemodialysis has no consistent effect on serum prolactin. However, without exception, serum prolactin levels return to normal following successful kidney transplantation.

The effect of estrogens on prolactin secretion has been referred to briefly before. While there may be a slight difference in basal prolactin levels between the sexes (37), additional studies will be required to define the extent of the differences more precisely, especially in view of the diurnal and other fluctuations. Female patients who receive estrogens in small amounts, 10–20 μg as in the "pill," generally have prolactin values in the normal range, although as a group the mean concentration may be slightly greater than normal (74). Moreover during the cycle any differences in serum prolactin in the follicular and luteal phase is very small if it exists at all. However, despite the lack of any major differences in basal values, under at least 3 circumstances clear sex differences in prolactin release can be distinguished. Females have a considerably greater TRH-induced PRL release, stress and anesthesia cause a greater PRL release in the female, and nipple stimulation also releases more prolactin in the nonpregnant female than in the male (74). These greater serum prolactin concentrations in the female probably reflect at least in part the greater prolactin content found in pituitaries from females. Finally, the ectopic production of prolactin by malignant tumors has been reported in a number of patients (75).

## PROLACTIN AND CANCER

There is a great deal of experimental work in mice and rats which implicates prolactin as an important hormone in the genesis of mammary cancer. This evidence has been reviewed by Furth (76), Meites (77), and Pearson (78). From these studies it would appear that prolactin acts as a promoter or cocarcinogen in the development of breast cancer. The evidence for this conclusion can be summarized briefly. After the induction of mammary tumors by dimethylbenzanthracene (DMBA) the administration of prolactin decreases the latent period of the appearance of tumors and increases the incidence and growth rate of the tumors. After adrenalectomy, oophorectomy, and hypophysectomy, hormone-responsive tumors regress, but will recur after the administration of prolactin, whereas estrogen administration has no effect. Increasing endogenous prolactin levels with chlorpromazine increases the incidence and growth of the tumors, while lowering serum prolactin with ergot drugs decreases the incidence of DMBA-induced tumors and the incidence of spontaneous mammary tumors in mice (79).

Furth showed that when a subcarcinogenic dose of 3 types of carcinogens—chemical, radiation, or tumor virus—are given to mice along with a subcarcinogenic dose of prolactin, the combined treatment (i.e. carcinogen and prolactin) resulted in an incidence of mammary tumors ranging between 40–60%. Recent reports suggest that tumor extracts from patients with breast cancer contain reverse transcriptase (80), an enzyme found in some oncogenic viruses. Furthermore, an antigen present in tumor extracts of some patients with breast cancer cross-reacts with antibodies to the mouse mammary tumor virus (81). These findings suggest that the mouse mammary tumor model may be a very good model for studies on human mammary tumor.

Currently, many investigators are actively engaged in studies designed to explore the role of prolactin in human breast cancer. It is clear that hypophysectomy is at least as beneficial as adrenalectomy in patients with metastatic breast cancer. Some hold that this may be due to a reduction of serum prolactin levels. However, some patients also go into remission after pituitary stalk section, a procedure which may be associated with elevated serum prolactin (82). This observation led some investigators to conclude that prolactin cannot promote breast cancer because remissions follow an increase in serum prolactin. It must be remembered, however, that estrogens also have this paradoxical effect: oophorectomy or testosterone is beneficial in some patients while in others the administration of estrogens leads to a remission. We have begun studies to see whether we can demonstrate prolactin binding sites in normal and malignant mammary tissue and to see whether the presence or absence of prolactin receptors correlates with the clinical response to endocrine ablative procedures or with the presence or absence of estrogen receptors. Jensen has shown that the likelihood of a favorable clinical response to endocrine ablative procedures is good if the tumor tissue contains estrogen receptors (83).

In view of the beneficial effects which result from lowering serum prolactin levels in mouse and rat tumors, studies using L-dopa and ergocryptine have been started in human subjects with advanced breast cancer (40, 84, 85). Conflicting reports regarding basal serum prolactin levels in patients with breast cancer have appeared. Some investigators find the basal values to be somewhat higher in patients with breast cancer but more importantly L-dopa fails to suppress the serum prolactin levels to values less than 2–3 ng/ml as it does in normal subjects. Their results, however, are contrary to our own experience, but we do find that with the chronic administration of L-dopa one frequently encounters fairly normal serum prolactin levels at some point throughout the day. These observations suggest that because of its short duration of action L-dopa may not be the ideal drug for long term therapy; nevertheless, isolated reports are beginning to appear of objective remissions induced by its administration. If these preliminary reports are substantiated on a larger series of patients for a longer period of time, then there can be no doubt that PRL plays an important role in the development of breast cancer in man, as it does in mice and rats. Finally one cautionary note must be added; namely, in 20 patients with advanced metastatic breast cancer treated with an ergot derivative, CB-154, no remissions were observed (85). The reasons for the difference between the results using L-dopa and CB-154 are clear. Unfortunately, serum prolactin levels were not measured in the patients treated with CB-154, but one would have expected the levels of prolactin to be markedly reduced by the doses employed. In summary, the available evidence does not allow us to state unequivocally that prolactin is involved in the genesis or promotion of human breast cancer, but it compels us to examine the possibility with the utmost attention. We believe that if the preliminary reports of the beneficial effect of L-dopa in patients with breast cancer are widely confirmed, there can be little doubt that prolactin is an important hormonal factor in the development of breast cancer.

## The Physiological Role of Prolactin

The only established role for prolactin in human subjects is the initiation and maintenance of lactation. Additional hormones such as growth hormone, insulin, adrenal, and ovarian steroids are required for successful lactation in animals. In our studies we have noted that some female patients with marked elevations of serum prolactin concentrations have no galactorrhea, whereas other patients with normal prolactin levels have profuse galactorrhea. It is not entirely clear which additional hormones or factors are essential for successful lactation. When CB-154 is given postpartum serum prolactin declines and lactation promptly stops (del Pozo & Friesen, unpublished observations). The effect of prolactin and other hormones on a number of biochemical events in the mammary gland has been reviewed elsewhere (86).

Nicoll (87) has reviewed and classified the many biological effects of prolactin in a large number of species, but which of these many actions are applicable to man is uncertain. Quantitatively more prolactin is released by the pituitary per day than any of the other hormones secreted by this gland, but apart from the effect on the mammary gland, no prolactin effects have been established in man. Several possibilities which must be considered are an influence on growth and development in utero and during early infancy and an effect on salt and water metabolism, especially during fetal life. These two possibilities are suggested because in a fascinating study of Sinha et al (88) antiserum to prolactin administered in the newborn period was lethal or produced drastic impairment of growth in a large number of mice. These findings raise the possibility that prolactin may have much broader effects than those presently being considered. Secondly, the amniotic fluid-to-serum prolactin gradient is so very high in early fetal life that prolactin may have special significance at this period of development.

The increases in prolactin under a variety of circumstances such as stress and exercise in both male and female subjects suggest that the hormone may have a more general metabolic effect. Indeed no possible role for prolactin in males has been suggested with the possible exception that it may influence the growth of secondary sex organs such as the prostate and seminal vesicles. In the rat, prolactin increases the cholesterol-ester pool size of the testes (89), but there is no good evidence that hPRL has any effect on testicular function. We have found that some men with pituitary tumors and markedly elevated serum prolactin levels (1000–10,000 ng/ml) have a profound loss of libido. However, in the presence of somewhat low gonadotropin and testosterone levels it is impossible to ascribe the symptom solely to an increase in prolactin.

## SUMMARY

With the isolation of human prolactin the close homology of the amino acid sequence of hPRL and sheep prolactin has been established as well as the greater structural differences between hPRL and hGH or hPL. There is no longer any room for doubt about the existence of a separate human prolactin molecule. The development and application of specific and sensitive radioimmunoassays have enabled investigators to measure serum prolactin concentrations in a great variety of physiological and pathological conditions. Pharmacological tests have been developed for the functional evaluation of the hypothalamic pituitary prolactin axis. Despite great advances in our knowledge of the chemistry and secre-

tion of human prolactin, our understanding of the physiological role of prolactin in man is still very limited. Finally, the studies on prolactin and breast cancer are most exciting because they may provide a clue to the genesis and treatment of a common and frequently lethal disease.

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# METABOLISM OF KETONE BODIES BY THE BRAIN 7076

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Recent developments have necessitated revision of some long-established concepts of the brain's energy metabolism. The brain is one of the most metabolically active organs in the body; in adult man it constitutes only 2% of body weight but accounts for 20% of the total body basal oxygen consumption (1, 2). Most tissues exhibit considerable flexibility in the choice of foodstuffs that they extract from the blood and utilize for energy. In contrast, it has generally been believed that the normal adult brain is restricted obligatorily and almost exclusively to glucose as the substrate for its energy metabolism (1, 2). This view must now be modified as evidence accumulates that in certain conditions and during certain periods of life the brain can and does rely, at least in part, on other substrates, particularly ketone bodies.

## METABOLIC SUBSTRATES OF NORMAL ADULT BRAIN

*Normal cerebral glucose utilization.*—The rates of cerebral oxygen consumption and glucose utilization in normal, conscious, young adult men are ~156 and 31 μmole/100 g tissue/min, respectively (1, 2). $CO_2$ production is essentially equivalent to $O_2$ consumption, and the cerebral respiratory quotient (RQ) is therefore close to unity (1, 2). An RQ of 1.0 indicates that the $O_2$ is consumed and the $CO_2$ produced in the oxidation of carbohydrate. The cerebral oxygen consumption is equivalent to 26 μmole glucose/100 g/min on the basis of 6 μmole $O_2$ consumed per μmole glucose completely oxidized to $CO_2$ and $H_2O$. Since the measured rate of glucose utilization is 31 μmole/100 g/min, there is more than enough glucose consumed to account for all the $O_2$ consumption. The excess glucose is probably distributed in various intermediates of carbohydrate metabolism, each of which is released into the blood in amounts insufficient to lead to detectable cerebral arteriovenous differences under normal circumstances. No positive cerebral arteriovenous difference, indicating net uptake by brain, has been found for any other potential substrate for oxidative metabolism in adult brain in normal conditions (1, 2). The combination of a cerebral RQ of unity, an almost stoichiometric relationship between oxygen and glucose consumption for the complete oxidation of the latter, and the absence of any significant arteriovenous difference for any other energy-rich substrate constitutes strong evidence that the normal adult brain derives its energy almost solely from the oxidation of glucose.

*Obligatory utilization of glucose.*—The fact that brain normally derives almost all its energy from aerobic oxidation of glucose does not distinguish between preferential and obligatory utilization. Most tissues are facultative in their choice of substrates and use them interchangeably roughly in proportion to their availability from the blood. It was once believed that the brain did not enjoy such flexibility and that its energy metabolism was rigidly restricted to the substrate glucose (1, 2). The basis for this conviction rested on two lines of evidence: 1. the impairment of cerebral function and energy metabolism by glucose deprivation, and 2. the inability of potential substrates other than glucose to reverse the effects of hypoglycemia.

Hypoglycemia is associated with changes in mental state ranging from mild, subjective sensory disturbances to convulsions, coma, and death, depending on both the degree and duration of the condition. The behavioral effects are paralleled by abnormalities in the electroencephalogram and cerebral metabolic rate, and cerebral oxygen consumption declines in proportion to the depression of the level of consciousness (1, 2). The cerebral effects of hypoglycemia are independent of the mode of its induction and are similar whether it is caused by insulin or hepatectomy (1, 2). All the effects are rapidly reversed by glucose administration, provided they have not been allowed to persist until irreversible changes have occurred. It appears then that there are no other substances normally present in blood which can replace glucose as the substrate for cerebral energy metabolism.

The hypoglycemic state provides an ideal situation to test substances for their ability to substitute for glucose in cerebral energy metabolism. A suitable substrate would be expected to restore consciousness and reverse the abnormalities in cerebral electrical and metabolic activities without raising the blood glucose level. Many potential substrates have been tested in man and animals. Few have been found effective, and of these all but one appear to operate by raising the blood glucose level rather than by serving directly as a substrate for cerebral metabolism (3). Mannose appears to be the only substance which can be utilized directly and sufficiently rapidly by the brain to restore normal function in the absence of glucose (4). It traverses the blood-brain barrier and is converted to mannose 6-phosphate by hexokinase which phosphorylates mannose as effectively as glucose. Mannose 6-phosphate is then isomerized to fructose 6-phosphate by phosphomannoseisomerase, which is quite active in brain tissue (5). It is through this pathway that mannose enters the glycolytic pathway and replaces glucose. Maltose is occasionally effective but only by raising the blood glucose level through its conversion to glucose by maltase activity in blood and other tissues (3, 4). Epinephrine produces arousal from insulin coma, but by stimulation of glycogenolysis and elevation of blood glucose concentration. Glutamate, arginine, glycine, *p*-aminobenzoate, and succinate are also occasionally effective, but probably through the release of epinephrine, which in turn raises the blood glucose level (3). Many substances tested and found ineffective are compounds normally formed and utilized within the brain and are normal intermediates in its intermediary metabolism. Lactate, pyruvate, fructosediphosphate, acetate, β-hydroxybutyrate, and acetoacetate are such examples (1–3). These can all be utilized by brain slices, homogenates, or cell-free fractions, and the enzymes for their metabolism are present in the brain. In some cases, e.g. glycerol (6, 7) or ethanol (8), the enzymes may not be present in amounts sufficient to carry out the high rate of utilization required by the brain. In some cases, e.g. D-β-hydroxybutyrate and acetoacetate (9), the enzymes are adequate, but the substrate is not available to the brain because of inadequate blood level or restricted transport through the blood-brain barrier. The brain requires continuous delivery of substrate by the blood, and there is thus far no known endogenous substance present in blood that can fully substitute for glucose. Mannose, the one substance found to be a suitable replacement, is not a significant constituent of blood. Glucose must therefore be considered essential for normal physiological and biochemical functioning of the nervous system.

## KETONE BODY UTILIZATION BY BRAIN IN VIVO

Recent evidence indicates that the brain is more flexible in its choice of metabolic substrates than once supposed. Although there is still no convincing evidence that it can function entirely without glucose, it can and does in some naturally occurring conditions substitute other substances, at least in part, for glucose. The ketone bodies D(−)-β-hydroxybutyrate and acetoacetate are such substances, and the brain utilizes them in a variety of ketotic states in proportion, more or less, to their concentrations in blood. These ketone bodies, which are interconvertible into one another, are byproducts of the metabolism of fatty acids (Figure 1). Their levels in blood are normally very low, but their rate of production is enhanced when fatty acid degradation is increased, presumably because of increased acetyl-CoA and decreased free CoA levels resulting from accelerated fatty acid degradation. Their primary source is liver which can produce but not utilize them because it lacks the enzyme succinyl-CoA:3-oxoacid-CoA transferase to convert free acetoacetate to acetoacetyl-CoA, a necessary step in the utilization of ketone bodies (Figure 1). As the liver increases its rate of production of ketone bodies in association with increased fatty acid metabolism, it releases them into the blood for utilization by other tissues.

*Starvation.*—Evidence that the brain can extract and utilize ketone bodies from the blood was first obtained in studies of man during starvation. Owen et al (11) studied three patients being treated for severe obesity by complete and prolonged fasting. Their total body carbohydrate stores at the onset of the fast were estimated to be 150–300 g, and metabolic balance studies indicated that their maximum rate of gluconeogenesis from protein and glycerol during the fast could not have exceeded 33 g/day. Since cerebral glucose utilization is normally ~110 g/day, the total body's glucose reserves were clearly insufficient to satisfy the brain's normal glucose requirements for more than a few days. Nevertheless, after 6–7 weeks of starvation these patients exhibited none of the usual signs of

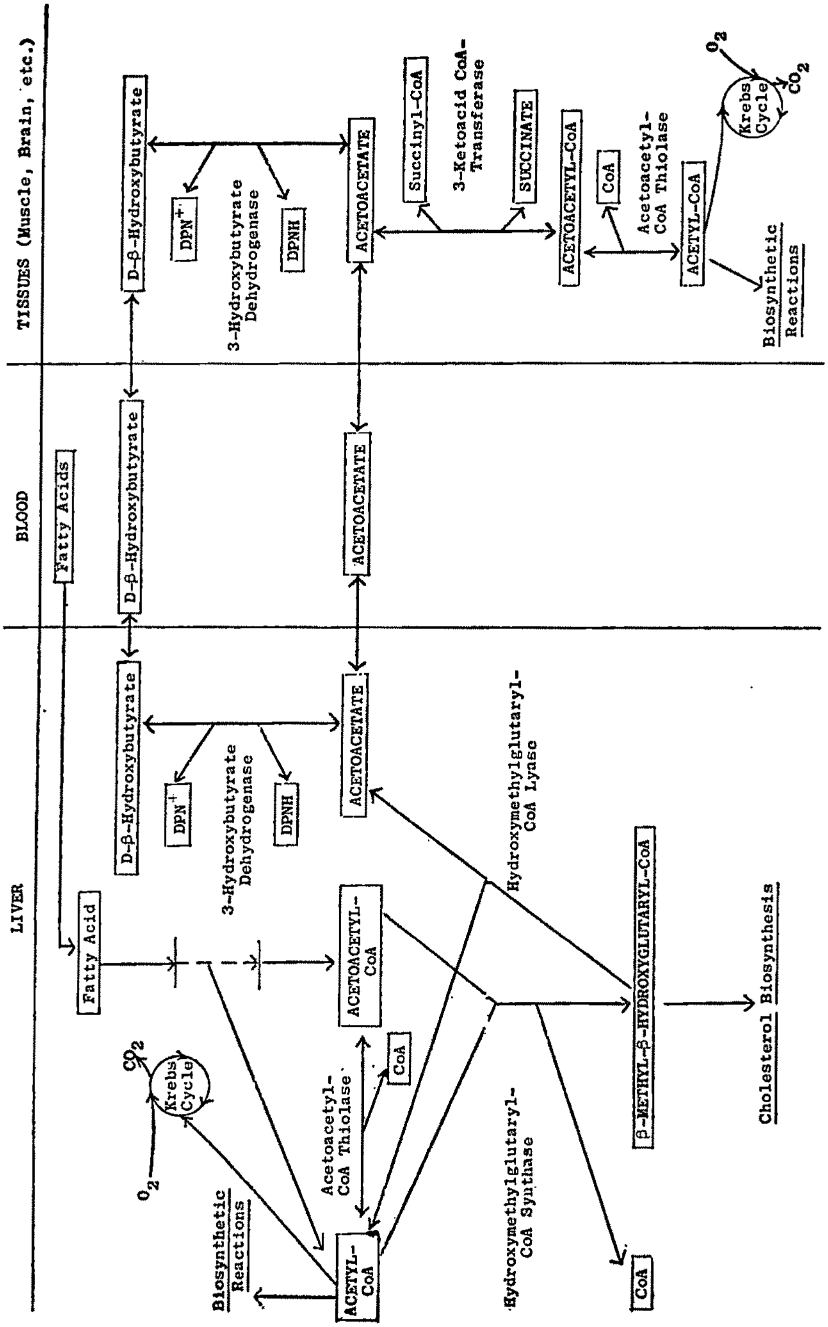

FIGURE 1. Metabolic pathways and enzymes of ketone body formation and utilization.

cerebral dysfunction indicative of cerebral glucose insufficiency. Their level of consciousness, EEG, and performance on psychometric testing remained within normal limits, evidence of normal rates of cerebral $O_2$ consumption. The cerebral arteriovenous differences identified the substrates for cerebral energy metabolism. The glucose arteriovenous difference had declined to about half normal level, and of this about half was recovered as lactate and pyruvate and did not support oxidative metabolism. Net lactate and pyruvate production by brain is normally hardly detectable (1–3). The arteriovenous $O_2$ difference was normal, and glucose utilization, after correction for lactate and pyruvate recovery, accounted for only 30% of it. Most of the remainder of the $O_2$ consumption was apparently supported by D-β-hydroxybutyrate and acetoacetate uptake, which could account for 52 and 8% respectively. Ten percent of the $O_2$ consumption remained unaccounted for. The higher rate of utilization of β-hydroxybutyrate than of acetoacetate reflected its higher concentration in the arterial blood. The levels of both ketone bodies were markedly elevated, but the β-hydroxybutyrate level was approximately 6 times that of the acetoacetate, the usual picture in ketosis. Krebs and associates (9) have shown in animals that the rates of utilization of ketone bodies by brain are in almost direct proportion to their concentrations in the arterial blood. Another interesting observation made by Owen et al (11) in their starving patients was the remarkably low cerebral RQ of 0.63. None of the substrates being oxidized could account for so low an RQ, which suggests that there is considerable $CO_2$ fixation and/or gluconeogenesis within the brain during starvation.

*Infancy.*—Like the human infant, the newborn rat is transiently hypoglycemic after birth, and its blood ketone levels are initially very low, as low as those of the normally fed adult rat (9). With the onset of suckling the blood ketone levels rise as much as tenfold, and a true ketosis ensues (9). This ketosis is of nutritional origin; it reflects the ketogenic nature of maternal rat milk which consists of 50% fat on a dry weight basis (12). The ketosis lasts until about 20–22 days of age when the rat is weaned onto standard high carbohydrate food; the ketosis then gradually disappears (9). During the ketosis of the suckling period ketone bodies constitute a significant source of the brain's substrates, and positive arteriovenous differences proportional to the concentration of the ketone bodies in the arterial blood are observed (9, 13). Similar evidence for significant cerebral ketone utilization during normal infancy has been obtained in canine puppies (14) and human infants (15). As discussed below, the enzymes of ketone utilization in brain are more active in early postnatal than in adult life; therefore, the infant brain is more efficient than the adult brain in extracting and utilizing ketone bodies from the blood at any given arterial concentration (9, 13).

*Miscellaneous ketotic states.*—The brain can utilize D-β-hydroxybutyrate and acetoacetate whenever they are available but does not normally do so in adult life because of their low concentrations in blood. Thus, hyperketonemia of various origins leads to significant utilization of ketones by brain. For example, fat-feeding or the infusion of D-β-hydroxybutyrate or acetoacetate, which raises the blood levels of one or both ketone bodies, leads to uptake of the ketones by the brain in approximate proportion to their levels in the arterial blood (9, 13, 14). There has thus far been no demonstration of cerebral ketone utilization in diabetic ketoacidosis. In fact, studies by Kety and associates (1) revealed no significant cerebral arteriovenous differences for total ketones in diabetic acidosis or coma. It may be that diabetic ketosis is an exception, possibly because of the hyperglycemia and markedly reduced cerebral metabolic rate associated with that condition (16).

## ENZYMES OF KETONE UTILIZATION IN BRAIN

The pathways and enzymes of ketone utilization are illustrated in Figure 1. Two enzymes, 3-ketoacid-CoA transferase and acetoacetyl-CoA thiolase, are required to metabolize acetoacetate to acetyl-CoA, the first intermediate in common with the pathway of glucose metabolism and that which enters the tricarboxylic acid cycle for ultimate oxidation to $CO_2$ and $H_2O$. The metabolism of D-β-hydroxybutyrate requires one additional enzyme, 3-hydroxybutyrate dehydrogenase, to oxidize it to acetoacetate, which is then metabolized as above. All three enzymes are widely distributed in mammalian tissues (9, 17–20), although the 3-hydroxybutyrate dehydrogenase, present in highest amounts in rat liver (17), is reported to be absent in the liver of ruminants (21). Also, mammalian liver generally lacks the transferase (10) and is therefore incapable of metabolizing ketone bodies. The dehydrogenase in liver functions, therefore, mainly in the reverse direction to form D-β-hydroxybutyrate from acetoacetate (Figure 1). The equilibrium constant probably favors this direction, and D-β-hydroxybutyrate is found in two- to sixfold higher concentration in blood than acetoacetate, even in marked ketosis (9). It is therefore D-β-hydroxybutyrate that is predominantly utilized by the brain in ketotic states (9, 11).

The maximum capacities of these enzymes are unknown in human brain, but in adult rat brain the maximum velocities of all three are comparable. The thiolase is present in small excess, and the transferase appears to be rate-limiting (9). The maximum possible rate of acetyl-CoA formation from either D-β-hydroxybutyrate or acetoacetate can be estimated to be about 80 μmole acetyl-CoA/100 g tissue/min in adult brain. Normal cerebral glucose consumption generates ~60 μmole acetyl-CoA/100 g/min. The enzymes of ketone utilization are thus presumably present in amounts sufficient for ketone bodies to meet the cerebral energy demands in the complete absence of glucose. This is yet to be demonstrated. Indeed, there is evidence to the contrary; in the perfused rat brain Sloviter (22) has found that complete replacement of glucose with D-β-hydroxybutyrate results in just as rapid deterioration of cerebral functional and metabolic activities as with complete removal of all substrate. A possible explanation of the inability of the ketones to substitute for glucose completely may be in the nature of the transferase reaction (Figure 1). In this reaction acetoacetate displaces the succinyl moiety of succinyl-CoA to form acetoacetyl-CoA. Succinyl-CoA hydrolysis is normally coupled to guanosine triphosphate (GTP) generation, and GTP has important functions in tissue metabolism, including protein synthesis and gluconeogenesis. It is possible that exclusive dependence on the utilization of ketones depletes the succinyl-CoA and GTP levels in brain and that some glucose utilization is required to maintain them at adequate levels. Indeed, Itoh & Quastel (23) have found evidence with brain slices that glucose accelerates the utilization of acetoacetate, probably by providing more succinyl-CoA.

The enzyme levels in brain do not remain constant throughout life but vary markedly with the postnatal maturation of the brain. The developmental patterns have been extensively studied in the rat. 3-Hydroxybutyrate dehydrogenase, an enzyme of the inner mitochondrial membrane (17), is present in small amounts at birth but increases along with many other mitochondrial enzymes in early postnatal life (9, 24, 25). This early rise probably reflects the increasing content of mitochondria in brain tissue during this period of life. The rise continues until a maximum, approximately 5 times the level at birth, is reached between 20 and 25 days of age. Then, in contrast to most other mitochondrial enzymes, which level off and remain constant into and through adult life, the 3-hydroxybutyrate dehydrogenase gradually declines almost to the low levels present at birth (9, 24–26). The 3-ketoacid-CoA transferase exhibits an almost identical pattern of development (9, 27), whereas the acetoacetyl-CoA thiolase level is maximal at birth, remains more or less constant during the first 30 days of life, and then gradually declines to a level in adulthood about two thirds that of infancy (9, 28). The different developmental pattern of the thiolase may reflect its functions in metabolic pathways other than that of ketone body utilization. The dehydrogenase and transferase act directly on the free ketone bodies, and their developmental pattern in brain closely follows the changes in blood ketone levels. Their levels rise with the increasing ketosis that suckling rats develop from their ketogenic diet and decline after weaning and the dissipation of the ketosis. The levels of these key enzymes of ketone body utilization in the brain thus appear to be closely adjusted to the levels of available substrate. The mechanism of this adjustment is unknown. Whether it reflects induction of enzyme synthesis by the ketosis, protection of the enzymes from degradation by saturation with their substrates (29), or merely a fortuitous correlation remains to be determined. There is controversial evidence to suggest that ketosis may play a role in the mechanism of the enzyme changes in brain. In one study maternal starvation and fasting at birth accelerated the rise of 3-hydroxybutyrate dehydrogenase activity in the brain of fetal and neonatal rats (30), but in another study no such effects were observed (25). Prolongation of ketosis following weaning by starvation or fat-feeding delays the decline in brain dehydrogenase activity that normally occurs at this time of life (26, 31), and premature weaning onto a high carbohydrate diet has been reported to initiate an earlier decline in the enzyme level (31).

Neither starvation (9, 27) nor fat-feeding (9, 32) appears to alter brain transferase activity at any age after birth, although a maternal high-fat diet has been reported to increase both the transferase and thiolase activities of fetal rat brain (19). Despite one report of induction of 3-hydroxybutyrate dehydrogenase in brain by starvation (33), which could not be confirmed even in the laboratory of origin (9, 20, 25, 26, 34), it is clear from numerous studies (9, 20, 25–28, 30, 32, 34) that ketosis does not lead to increased activity of the enzymes of ketone utilization in adult brain.

## CONCLUSIONS

The normal adult brain utilizes glucose almost exclusively as the substrate for its energy metabolism, but, contrary to previous beliefs, it is not obligatorily limited to glucose. Studies with brain slices in vitro have shown that mature and immature brain, particularly the latter, has the enzymatic machinery to oxidize ketone bodies (23, 35). Recent studies in man and animals have shown that the brain in vivo turns in special circumstances to D-β-hydroxybutyrate and acetoacetate as its main substrates. The ketone bodies serve not only as sources of energy derived from their oxidation to $CO_2$ and $H_2O$ but also contribute carbon residues to constituents of brain normally dependent on glucose metabolism, for example, phospholipids, cholesterol (14), and the amino acids glutamate, glutamine, aspartate, and γ-aminobutyric acid (36).

Ketone utilization is regulated in adult brain entirely by the supply of substrate brought to it in the blood. The concentration of ketone bodies in the blood is normally very low, and cerebral ketone utilization is then negligible. In ketotic states caused, for example, by starvation or high-fat ketogenic diets, the brain utilizes ketone bodies in almost direct proportion to their concentrations in the arterial blood (9, 13, 37). Their rate of utilization may then exceed that of glucose. There is no evidence that they can totally replace glucose; indeed, the complete substitution of D-β-hydroxybutyrate for glucose causes as rapid a deterioration of cerebral function as the removal of all substrate (22). There is evidence that in diabetic acidosis and coma the brain may not increase its ketone utilization, despite the marked ketosis, perhaps because of the reduced cerebral metabolic rate and the elevated blood glucose levels (1, 16).

Significant rates of cerebral ketone utilization are normal in early postnatal life; contrary to the situation in adult life, regulation is achieved not only by substrate supply but also by modification of enzyme levels. The newborn is slightly hypoglycemic and becomes ketotic as a result of the high-fat content of maternal milk. This is particularly true in the rat, although cerebral ketone utilization has been observed in human infants as well (15). Two key enzymes of ketone utilization increase in the brain throughout the suckling period and then decline again following weaning. Whether or not the enzymes can be artificially induced by ketosis is still uncertain for early life but clearly does not occur in the adult.

The brain is, therefore, not the inflexible selective organ it was once thought to be, but shares with many other organs the capacity to adapt to changes in its nutrient supply. Glucose still appears to be its preferred substrate and may even be essential to some degree, but when the brain's glucose supply becomes endangered and/or ketone bodies become more available, it can turn to ketone bodies for a significant fraction of its energy supply.

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# CURRENT CONCEPTS IN THE TREATMENT OF CHRONIC MYELOCYTIC LEUKEMIA

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There has been little change in the treatment of chronic myelocytic leukemia in the generation since the introduction of busulfan (Galton 1), and the great majority of patients today are treated with this drug using dosage schedules developed 20 years ago. The principal advances in more recent years include improved definition of the natural history of the disease and its various biochemical and pathological features, discovery of the Philadelphia chromosome and general application of karyotypic analysis, identification of features which have significant prognostic import (Theologides 2), and introduction of various additional chemotherapeutic agents. Excellent remissions can now be induced by any of several drugs in addition to busulfan, and more cumbersome methods of treatment, such as splenic irradiation or systemic administration of radioactive phosphate, have been abandoned.

The past 20 years have seen substantial improvement in the quality of disease control; most patients under treatment today are asymptomatic and fully functional throughout most of their course. However, survival has remained disappointingly poor, and it is very doubtful whether any prolongation of life can be attributed to antileukemic chemotherapy. Ironically, the modest improvement in survival recorded by the End Results Group (Cutler 3) during the past 20 years is less than that in chronic lymphocytic leukemia, in which comparable hematologic remissions are uncommon. This failure to achieve prolongation of survival commensurate with the quality of the remissions induced is reminiscent of early experience with the chemotherapy of Hodgkin's disease (Gellhorn & Collins 4), but is unique for modern treatment of neoplastic disease.

## CONVENTIONAL THERAPY AND COURSE

Galton (5) recently presented an excellent summary of generally accepted teaching about myelocytic leukemia and its treatment. His views, based principally on British experience, are similar to those expressed by leading authorities in the United States and other countries. Busulfan is the preferred therapeutic agent and is used as long as possible. Galton recommends a maximal daily dose of 4 mg to avoid bone marrow aplasia. Most American authors are somewhat more aggressive, often using 6 mg per day for initial therapy (Haut et al 6), and sometimes slightly higher doses. With daily doses of 4–6 mg, three or four weeks of therapy are required before a decrease in white blood cell (WBC) count occurs.

Patients are treated either with interrupted courses of busulfan, or continuously. With the former technique, busulfan is administered daily until the WBC count reaches normal (Haut et al 6) or near-normal (Galton 5) levels. Therapy is then withdrawn until the leukocyte count rises to some predetermined value, e.g. 50,000/mm$^3$. A second course of busulfan is then given, and treatment is again discontinued when the WBC count reaches the desired range. This process may be repeated a number of times. In almost all cases in which this scheme of treatment is used, the longest unmaintained remission is that following the first course of busulfan. Subsequent remissions become progressively shorter until it is no longer worthwhile to use interrupted therapy. Patients are then placed on continuous busulfan treatment, with doses of the drug adjusted to maintain the WBC count between 10,000 and 15,000/mm$^3$.

Interrupted therapy results in a total duration of exposure to busulfan that is considerably less than if continuous therapy were used and thus provides protection against toxic effects associated with long-continued administration of this drug such as skin pigmentation, disorders of the reproductive system, pulmonary fibrosis, and a wasting syndrome resembling adrenal cortical insufficiency (Galton 5, Oliner et al 7, Kyle et al 8, Feingold & Koss 9). Advocates of continuous therapy believe that it is undesirable to allow overt relapse of leukemia with its risk of recurrent symptoms, increase of spleen size, and possible development of manifestations difficult to reverse. Continuous therapy appears superior in maintaining good clinical control and normal hemoglobin (Galton 5), but I know of no study in which intermittent and continuous therapy were compared for effect on survival. We prefer continuous therapy, with complete suppression of leukocytosis. We have found that although many patients are not symptomatic at WBC counts of 30,000–50,000, many others are. We have seen a few patients who consistently feel worse with even slight leukocytosis (e.g. WBC of 12,000/mm$^3$) than when their WBC count is fully controlled.

In new patients, we discontinue busulfan when initial remission is achieved because of the prognostic information this affords. Bergsagel (10) has demonstrated that there is good correlation between the duration of remission following the first course of busulfan and total survival from diagnosis. His data show a straight-line relationship between the "doubling time" of the WBC count after treatment is discontinued and survival. He and Galton (5), believe that a stable leukocyte doubling time can be defined during a period without treatment. This appears to be true in many cases but is not a uniform finding. We have seen a number of patients whose course after discontinuation of busulfan consists of three distinct phases: 1. a normal WBC count, maintained for a significant period of time; 2. a period of mild and fluctuating leukocytosis, often with transient decreases of the WBC count into the normal range; and finally, 3. a period of relatively rapid increase in the WBC count, which *can* be characterized by a doubling time. Figure 1 illustrates an example of this pattern in one of our longest

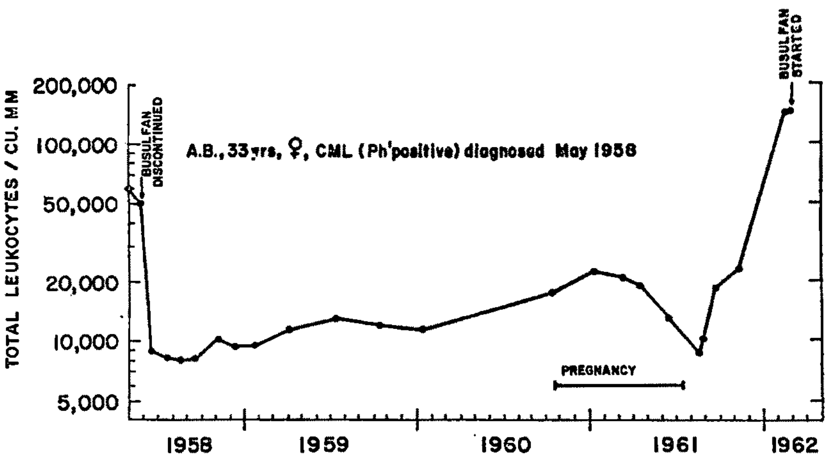

FIGURE 1. White blood cell count of a patient with chronic myelocytic leukemia after her first course of busulfan during 45 months without therapy. Note the mild leukocytosis (probably aggravated by pregnancy) which persisted for over 2 years before an exponential rise in WBC count occurred.

survivors with myelocytic leukemia. The second phase was unusually long in this case. Djaldetti et al (11) described a patient in whom the first phase—normal WBC count—lasted for five years, followed by about a year of fluctuating WBC count, and then a rapid rise to 150,000/mm$^3$. Haut et al (6) also observed this three-phase pattern in some of their patients. Obviously, a long period before onset of the exponential increase in the leukocyte count augurs well for the patient. Of particular interest to us is the phase of mild and fluctuating leukocytosis, which we interpret as a period during which proliferation of leukemic cells is substantially inhibited by host mechanisms.

A variety of effective drugs that are useful alternatives to busulfan under various circumstances have been described. For example, when busulfan induces thrombocytopenia, substitution of a drug with less platelet-depressing effect often permits control of leukocytosis to be maintained while the platelet count rises gradually. Table 1 presents a partial list of such alternative drugs of different chemical classes and varying mechanisms of action. The oldest of these, 6-mercaptopurine, has been used principally in acute leukemia, but is quite effective in chronic myelocytic leukemia and it has less platelet-depressing effect than busulfan (Shullenberger 12). Uracil mustard is also an effective drug (Frommeyer 13) and may be useful in cases with thrombocytosis resistant to busulfan (Robertson 14). Kennedy (15) ranks hydroxyurea as equal to busulfan for general use, but most therapists would consider it inferior as it has a higher incidence of unpleasant side effects and does not provide as smooth control. Dibromomannitol has been used in Europe for several years (Ramanan & Israels 16), but is still an investigational drug in the United States; it is often effective in busulfan-resistant cases. Dibromodulcitol, more recently introduced, may prove to be somewhat better (Sellei et al 17). None of these agents can induce long unmaintained remissions and thus continuous treatment schedules are used. They usually do not provide as stable control of leukocytosis as busulfan, but may be effective, for varying periods of time, in patients who have become resistant to busulfan.

Survival with chronic myelocytic leukemia has not been accurately defined. Most hematologic groups report median survivals from diagnosis of 2½ to a little over 3 years (Galton 5, Grifoni et al 18). However, Feinleib & MacMahon (19) presented evidence that actual survival is very much shorter than this; they believe that many patients who die early do not appear in reports from major hematologic centers for a variety of reasons. Perhaps the most accurate figures are those reported by the End Results Group, which collects data from over 100 hospitals in several states. This group recorded a 3-year survival rate of 26 % for patients diagnosed during 1960–1964 (Cutler 3).

TABLE 1. Some Alternative Antileukemic Drugs

| Agent | Mechanism of Action. | Special Features |
|---|---|---|
| 6-Mercaptopurine | Inhibits nucleic acid synthesis | Weaker platelet depressor than busulfan; useful in thrombocytopenic patients |
| Uracil mustard | Alkylating agent | More potent platelet depressor than busulfan |
| Hydroxyurea | Inhibits DNA synthesis | Very rapid action (2 days) |
| Dibromomannitol, dibromodulcitol | Alkylating agents | Representatives of an interesting new class of drugs |

## Common Complications

Myeloblastic transformation is the most common terminal complication of chronic myelocytic leukemia, occurring in about two thirds of all cases. Until recently it was almost always irreversible, and resulted in death within 1–6 months. During the past few years, some success has been recorded in reversing blastic transformation, but remissions are usually brief. Various schedules of combination chemotherapy are used, similar to those employed in acute myeloblastic leukemia. Effective therapy of this complication requires the facilities of a major hematologic center.

We have found splenectomy useful in patients with persistent splenomegaly and splenic infarction, mechanical symptoms, thrombocytopenia or anemia, and in some cases of blastic transformation (Sokal, Elias & Mittelman 20). In the hands of an experienced hematologic-surgical team, splenectomy can be performed safely even in acute blastic crisis and occasionally may contribute to a long-lasting remission.

Basophilia is a common finding in Philadelphia chromosome-positive leukemia. When peripheral basophils are persistently above 20%, the prognosis is poor (Ezdinli et al 21). Sometimes basophilia can be controlled by changing the therapeutic agent, but often it proves irreversible.

### NEW APPROACHES TO THERAPY

Conventional therapy of chronic myelocytic leukemia is deliberately palliative. It has been assumed that survival cannot be prolonged by the therapeutic modalities currently available and therefore that there is no justification for subjecting patients to the inconvenience and risks of more intensive therapy. This may well be true, but it has not yet been proven to my knowledge. The danger of assuming that survival cannot be prolonged has been well demonstrated in Hodgkin's disease; quality of life was improved by palliative therapy, but survival was not affected until some therapists set cure as their goal and undertook intensive radiotherapy or combination chemotherapy, accepting considerable toxicity from these therapeutic schedules.

There is now a willingness to depart from the traditional palliative techniques of treatment. Many hematologists attempt to suppress leukocytosis completely, rather than accepting a "slightly leukemic" blood count. Some are changing chemotherapeutic agents at arbitrary time intervals, *before* resistance develops. We have explored combination chemotherapy using pairs of drugs with different mechanisms of action, and attempted to achieve and maintain not only a normal WBC count (4000–8500/mm$^3$) but also a normal bone marrow aspirate. We have been only partially successful in achieving full marrow remissions and have no evidence yet that our relatively aggressive therapy will improve survival. We have learned, however, that excellent suppression of leukemia can be maintained by several combinations without clinically significant toxicity. In many cases, smooth control of the disease is much easier to maintain with two-drug combinations than with single-agent therapy. For example, a patient who develops thrombocytopenia during busulfan therapy and a fluctuating WBC count with thrombocytosis while receiving 6-mercaptopurine will often maintain normal and stable WBC and platelet counts when treated with a combination of the two drugs.

Some therapists are exploring much more aggressive treatment programs at the time of diagnosis in an attempt to eliminate the neoplastic clone of marrow cells early in the disease, while normal stem cells are still presumably present. One such program uses early splenectomy to remove a major reservoir of leukemic cells, followed by repeated cycles of combination chemotherapy with cytosine arabinoside and 6-thioguanine to destroy leukemic myeloblasts (Clarkson, personal communication).

Much effort is being devoted to identify biologic control mechanisms which regulate multiplication and maturation of granulocyte precursors in the hope that such natural regulators, if identified, could be used therapeutically in myeloid leukemia. Leukemic myeloblasts, which do not mature in the patient with terminal disease, may produce apparently normal granulocytes during in vitro

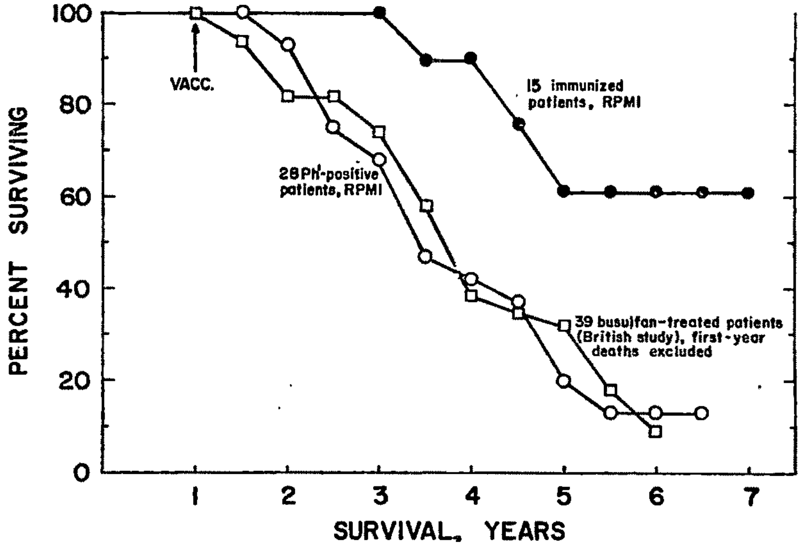

FIGURE 2. Survival of 15 immunized patients with uncomplicated Philadelphia chromosome-positive leukemia, compared with that of unimmunized patients treated at the Roswell Park Memorial Institute, and busulfan-treated patients from a recent British study. The immunized patients entered this program at varying times after diagnosis (median: 1.6 years). To simplify construction of the survival curve, it was assumed that each patient had had leukemia for 1 year at the time of initial vaccination. The control series were adjusted for this by eliminating first-year deaths.

culture (Robinson, Kurnick & Pike 22). Obviously, identification of the maturation factors operative in such in vitro cultures (or the factors which prevent maturation in vivo) should be useful.

Chalones, products of mature cells which inhibit proliferation of their precursors, are now receiving increasing attention. They are tissue- but not species-specific. Rytomaa & Kiviniemi (23) have treated Shay's chloroleukemia in rats with granulocytic chalone extracted from bovine leukocytes. They reported prolongation of survival in all animals and cure in 9 of 40.

Another approach to the management of chronic myelocytic leukemia is based on the assumption that immunologic defense mechanisms may inhibit proliferation of leukemic myeloblasts. We have attempted to stimulate such host defenses by repeated vaccination with mixtures of BCG organisms and cultured cells of lines established from blood of patients with myeloblastic leukemia or blastic crisis of chronic leukemia (Sokal, Aungst & Grace 24). If immunization is started when leukemia is under good control, substantial general stimulation of cellular immune mechanisms is regularly achieved, as well as strong delayed hypersensitivity to antigens of the target cells. Patients continue to need antileukemic chemotherapy, but myeloblastic transformation is inhibited, and survival is prolonged significantly (Figure 2).

## CONCLUSIONS

The past twenty years have seen considerable progress in our understanding of chronic myelocytic leukemia, introduction of several effective agents in addition to busulfan, improvement in the quality of disease control, and substantial reduction in the interference with patients' normal activities by this disease or its treatment. However, survival has not been significantly prolonged.

We now stand at the threshold of a new therapeutic era. Greater success in the management of myeloblastic transformation can be expected in the next few years. More intensive treatment and application of new modalities of therapy offer promise of substantially improving survival with chronic myelocytic leukemia.

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# PREVENTIVE ANTIBIOTIC MANAGEMENT IN SURGERY

7078

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The general view of bacterial infection prevalent in medicine today is concerned with the interaction of host and bacteria, rather than almost exclusively with bacteria as it was a few years ago following the introduction of antibiotic substances. This evolution, which promises to identify methods of infection prevention, has found vigorous support in surgery because a primary problem in this discipline is the prevention of bacterial disease rather than the elimination of an established septic problem. Postsurgical infection has always been a limiting factor in the development of surgery, and it is not surprising that surgeons greeted the advent of antibiotics 25 years ago with a vigor born of the expectation that the day of postoperative wound infection was over (1). In the period immediately following the introduction of penicillin and streptomycin, these antibiotics were used widely and indiscriminately in the hope that wound infection would be avoided. What effect this initial euphoria had on the efficiency with which aseptic technique or technical operative accuracy was carried out by surgeons is important but not pertinent here. What is relevant to this discussion is the disillusion following the realization that the new technique did not produce the expected results. The pendulum then swung rapidly from the position where all surgical patients were indiscriminately given antibiotics to the point where, with equal lack of discrimination, no surgical patient was given an antibiotic in an attempt to *prevent* a postsurgical infection. Recent information stemming from experimental work and the renewed interest in the host and his ability to defend himself has given a more accurate view of the problems of preventing postsurgical infection, and within the past year the pendulum has again begun to swing (2). There is also reason to believe that this traverse will be generated by an increase in the surgeon's ability to prevent postoperative bacterial complications. That is, the changes in preoperative management are based on current biologic information concerning the development of infection as related to the hope that antibiotic substances would eliminate bacteria from tissue at any time, place, or circumstance. It is the purpose of this writing to examine the biologic problems encountered in preventing the development of a bacterial infection in the surgical wound as well as to outline a program of clinical management designed to take advantage of the information available.

A discussion of the problems surrounding the prevention of postoperative wound infection must begin by acknowledging that the natural antibacterial mechanisms of the patient are by far the most important factor in preventing infection. This nonspecific host resistance against bacterial invasion is composed of a number of distinct factors, some of which operate systemically, while others operate only at the site of bacterial contamination. These host defensive mechanisms begin to act the instant bacteria arrive in the tissue. There is no lag, or grace period, between bacterial contamination and reaction (3). Recent studies of host resistance have demonstrated the immediate reaction to be a definite period of intense, effective antibacterial activity beginning at the moment of contamination and ending a few hours following the arrival of bacteria, even though the anatomic bacterial lesion continues to develop in the sense of classic inflammation for 20 hr or more (4). This early period of intensive activity, where the development of a bacterial lesion appears to be decided by the effectiveness of the patient's nonspecific antibacterial forces, has been termed by Miles the "decisive period in defense against bacterial invasion" (5).

If the natural antibacterial mechanisms of the patient are accepted as the major factor in preventing bacterial invasion, then any system designed to prevent infection should first insure that the host's defensive mechanisms are intact and functioning at the highest efficiency. Then, and only second, should one consider supplementing the host's native ability with an unrelated antibacterial activity during periods of high risk. Maintenance of host resistance is, for practical purposes, a problem of insuring the maintenance of normal physiology and need not be explored at length at this time. Supplementing the natural resistance of the patient at the time of unusual risk, however, is a relatively new concept and requires detailed explanation (2).

The patient undergoing surgery provides somewhat of a special case in the development of clinical infection for several reasons. First, the incision breaches the skin barrier allowing bacterial contamination of deep tissue; second, anesthesia and surgery provide physiological challenges to the patient which tend to disturb his normal physiology and decrease his usual resistance against bacterial invasion; and third, and perhaps for this discussion the most important, the exact time when both the mechanical barriers are broken and the patient's natural resistance is reduced is known well in advance and is of short predictable duration. Therefore if it were possible to assist the patient during this period of reduced ability to prevent bacterial infection, appropriate compensatory plans could be made and carried out. This is far different from the usual situation in clinical medicine where the time of bacterial contamination leading to infection is not predictable (i.e. onset of lobar pneumonia or pyelonephritis, etc). Further, since the outcome of interaction between host and contaminating bacteria, regarding the prevention or the development of bacterial lesion, is decided during the first few hours following bacterial contamination of tissue (3), the point at which supplementation is no longer needed can be accurately determined. A brief description of this evidence follows.

In order to test the concept that the major portion of nonspecific resistance against bacterial invasion is expended during the first few hours following contamination and further that the prevention or development of bacterial lesions is determined during this period, animal experiments were designed in which natural host mechanisms were blocked or supplemented at various times following reproducible bacterial contamination of tissue. The inoculum used in these experiments produced a localized, standard-sized lesion whose maximum size was reached at 24 hr. These experiments demonstrate the correctness of the hypothesis. If host defenses are blocked during the first 3 hr following bacterial contamination (decisive period), the 24 hr lesion size will be greatly increased. However, if the host defenses are blocked after the decisive period, the lesion size is the same as the controls, presumably because the lesion and its size had already been established. If the host defensive mechanisms are supplemented by an antibiotic during the decisive period, the lesion is prevented or markedly reduced in size (2). Again, if the antibiotic substance is delivered after the decisive period, the lesion is unchanged from the control, also presumably because the character of the lesion was determined during the decisive period.

Figure 1 graphically demonstrates the difference in lesion development in

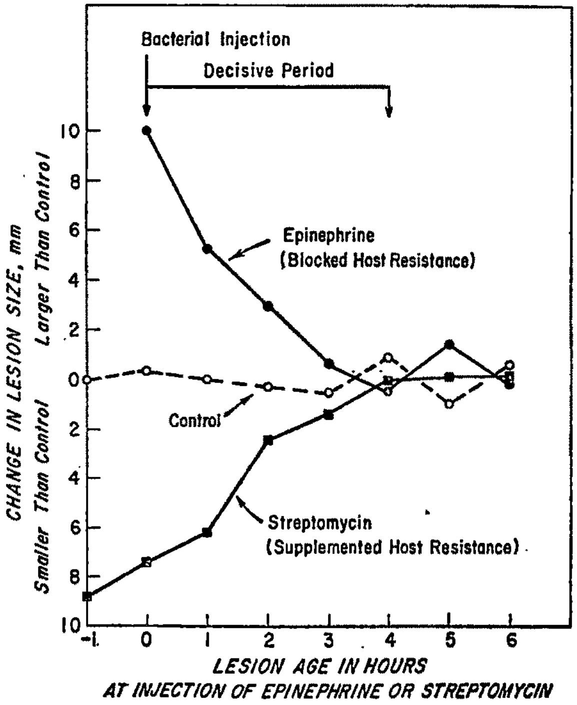

FIGURE 1. Variation in bacterial lesion size depending on functioning level of host resistance.

animals where host resistance has been blocked or supplemented. These experiments were carried out in guinea pigs whose tissues were contaminated by an intradermal injection of $2 \times 10^8$ viable *Pseudomonas* organisms, producing a 10 mm lesion in the control animal. Blocking was carried out at various times in relationship to bacterial contamination of tissue by causing local ischemia using adrenalin. The host antibacterial potential was supplemented at various times by a single intravenous injection of streptomycin. The curves represent the relation between maximum lesion size and the length of time between bacterial contamination and the onset of blocking or supplement to host resistance: they are mirror images of each other, both coinciding with the decisive period. Further, the maximum effect is achieved in either case if the host defensive mechanisms are blocked or supplemented before the tissue is contaminated with bacteria. Both effects slowly diminish as the time between bacterial contamination and the advent of blocking or supplement increases until approximately 4 hr after bacterial contamination. From this time onwards, attempts to supplement or inhibit antibacterial defenses have no effect on the lesion size which will develop.

The above experiments concerning the biology of infection establish several important principles that can be applied to the prevention of wound infection in clinical surgery. First, the effectiveness of defense against bacteria depends largely on natural resistance. Second, this resistance is reduced by the abnormal physiology induced by anesthesia and operation. Third, the risk of infection can be decreased and, in specific cases, infection prevented by supplementing the host's antibacterial resistance, but only if the supplement is delivered before bacterial contamination of the tissue so that it is available to supplement the patient's intrinsic efforts during the early decisive period. Fourth, supplements to host resistance serve no purpose if they are delivered for periods longer than 4 hr following the end of the period of active bacterial contamination.

Thus, the concept of *preventive antibiotics* in surgery can be stated as follows. *Preoperative, preventive antibiotics are indicated if there is a high probability that the patient's natural resistance to bacterial invasion will not overcome the combined bacterial and physiologic challenge posed by a surgical procedure. Further, the antibiotic substance used to supplement host resistance must be circulating at high concentration in the patient's tissue before contaminating bacteria arrive.*

The categories of patients who, for one reason or another, have a high probability of postoperative bacterial complications can be divided into three groups: (*a*) patients with a disease or therapeutic regimen that interferes with the normal function of one or more of the natural mechanisms of host resistance, (*b*) patients in whom the surgical procedure involves the hazard of massive bacterial contamination of previously noncontaminated tissue, (*c*) patients in whom the extensive nature of the procedure and/or the use of large volumes of foreign material threaten to overwhelm the ability of the host to defend himself.

Table 1 lists clinical examples of patients in Group *a* with altered host resistance. Table 2 lists clinical examples of patients in Group *b* threatened by massive bacterial contamination during operation.

The third group is difficult to define in precise clinical examples. However, two general categories can be mentioned: first, those patients undergoing operative procedures whose extent seriously compromises normal physiology; second, those patients undergoing operation incorporating the use of implantable foreign material and where sepsis might result in death or extensive morbidity. Certain cardiac, orthopedic, neurosurgical, and vascular operations fall in these groups.

**TABLE 1. Patients with a disease that interferes with the normal function of one or more of the natural mechanisms of host resistance**

1. Severe metabolic derangements such as those caused by:
    - Longstanding intestinal obstruction
    - Starvation
    - Diabetic acidosis
    - Cirrhosis of the liver
2. Disease or congenital defects involving normal bacterial defense mechanisms:
    - Agammaglobulinemia
    - Leukemia
    - Aplastic anemias
    - Radiation sickness
    - Hypersplenism
    - Hemolytic anemias
3. Diseases creating infection-prone areas:
    - Valvular heart disease
    - Congenital heart disease
    - Chronic pulmonary disease
    - Extensive radiation fibrosis
    - Vascular insufficiency
4. Bacterial diseases in patients whose natural or acquired resistance to the specific bacteria is low:
    - Tuberculosis
    - Fungus diseases
5. Corticosteroid therapy or disease producing high levels of corticosteroids
6. Large areas of tissue damage secondary to trauma

**TABLE 2. Patients in which the surgical procedure involves the hazard of a massive bacterial contamination**

1. Ruptured appendicitis
2. Gangrenous cholecystitis
3. Perforated diverticulitis or perforated carcinoma of the colon
4. Drainage of an abscess involving opening of tissue planes
5. Excision of localized empyema cavity
6. Decortication in the face of pleural sepsis
7. Excision of a brain abscess
8. Bacterial disease in the watershed of lymph nodes to be excised
9. Surgical excision of a tuberculous process
10. Debridement of traumatic contaminated wounds

The choice of antibiotic substance must be a clinical decision based on the state of the patient, the type of surgery to be carried out, and the nature of the hospital endemic bacterial population. In general, the most reasonable choice is a single antibiotic with a broad spectrum covering the range of bacterial species usually encountered during the operative procedure to be performed. In the unusual circumstances where the risk is high and both gram-positive and -negative species can be expected, two antibiotic substances of broad spectrum may be used.

A high level of antibiotic substance circulating in the tissue when bacterial contamination occurs is insured by delivering the antibiotic to the patient in sufficient time and dose. Both the intramuscular and intravenous routes have been successful. If the antibiotic is to be delivered by the intramuscular route, it must be administered about 1 hr before anesthesia is begun and repeated every 3 hr during the surgical procedure. If the antibiotic is to be delivered intravenously, it can be given immediately following the establishment of an intravenous line before anesthesia is begun in the operating room. Again, it should be repeated every 3 hr during the operation. For purposes of preventing infection, the antibiotic should be discontinued at the end of the period of increased risk. This can be accomplished by giving the final dose of antibiotic as the patient enters the recovery room. The matter of dose is not settled firmly. It appears reasonable at this time to deliver antibiotics over the preventive period at about the same dose used to treat serious, established infection. A total of three quarters of the daily recommended dose in increments of recommended size for the antibiotic to be used, depending on the length of operation, has provided excellent clinical results.

Clinical studies carried out in double-blind fashion on populations of surgical patients undergoing high risk operative procedures have demonstrated the effectiveness of *preventive antibiotics* (6–10). It is important to note that not only can the risk of postoperative infection be reduced by preventive antibiotics in appropriate patients, but the cost of antibiotic administration in allergic reactions, changes in endogenous or hospital flora, as well as in economics, are reduced well below the level encountered when one uses therapeutic antibiotics to treat an established infection.

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# RECENT ADVANCES IN EVALUATION OF GONADOTROPIC HORMONES

7079

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## Introduction

Over the past five years radioimmunoassays for gonadotropins have been applied extensively in studying control of gonadotropin secretion during physiologic and pathophysiologic states of human gonadal function. Results of these studies have been rather fully reviewed (1–3). Despite remarkable contributions to the understanding of endocrine control of gonadotropin secretion, results obtained with different reagents have not always been consistent (4). Furthermore, discrepancies have been noted when immunoassay potency estimates are compared with bioassay estimates, using a single reference preparation in both systems (5, 6). Frequently, it has been impossible to rationalize discrepancies though it has been supposed that they originated in variable structural requirements for biologic and immunologic binding sites (5).

In the following discussion, recently delineated chemical, biological, and immunological properties giving rise to these disparities will be reviewed. Exploitation of these findings has provided rational explanations for some discrepancies, facilitated the production of specific reagents, and extended the applications of gonadotropin measurements in humans.

## Chemical Properties of Gonadotropins

*General.*—There are three substances that stimulate growth and both gametogenic and steroidogenic function of ovary and testis in humans. Two of these, human luteinizing hormone (hLH) and human follicle stimulating hormone (hFSH), can be demonstrated in extracts of pituitary tissue, serum, and urine from normal humans after the fifth month of fetal life (7). The third, human chorionic gonadotropin (hCG), immunologically and biologically similar to hLH, is secreted by the trophoblastic epithelium of the placenta villus and is normally present in serum of mother and fetus during pregnancy and the immediate postpartum period (8). In addition, neoplasms of trophoblast and a variety of other anaplastic tumors secrete a substance indistinguishable from hCG (9).

All three hormones are glycoproteins consisting of a protein core with carbohydrate chains terminating in glycosidically linked sialic acid (N-acetylneuraminic acid) or fucose (10). The sialic acid content varies: hCG has 20 residues per molecule (11), hFSH has 5 (12), and hLH has only 1 or 2 (13).

*Sialic acid and biological and immunological properties of gonadotropins.*—The plasma half-life of gonadotropins with LH activity has been shown to be directly related to their metabolic clearance rates (14). Progressive, selective, enzymatic removal of the sialic acid residues, known as desialylation, results in proportional increase in metabolic clearance rates and reduction in biologic activity in vivo; the metabolic clearance rate and residual activity of completely desialylated hCG are less than 1% of those for native intact hormone (15, 16). In contrast, in an assay system based upon stimulation of testosterone secretion by rat Leydig cells in vitro, completely desialylated hCG retained about 25% of the activity of the fully sialylated hormone (17). Desialylation of hFSH in urine extracts (18) and hLH in pituitary extracts (19) results in substantial reduction of in vivo biologic activity of these substances as well. Results of in vivo and in vitro studies agree with the hypothesis that sialic acid protects gonadotropins, like other glycoproteins (20), from metabolic degradation but is not essential for target-organ recognition (21).

The role of desialylation in the metabolism of the glycoprotein hormones remains to be elucidated. Desialylation of hCG may occur in vivo since extracts of urine from pregnant women contain variably desialylated hCG molecules (22). It has not been determined whether desialylation occurred prior to secretion, during transit of the hormone from trophoblast to urine, or whether the entire carbohydrate side chain was synthesized incompletely in the cell of origin. Bridson et al (23) have shown variable immunologic/biologic activity ratios (*I/B*) of hCG secreted by different clonal strains of choriocarcinoma cells in vitro, suggesting that some variability arises during synthesis.

Effects of desialylation on antigenic potency of hLH, hFSH, and hCG were variable: while the potency of hLH and hFSH appeared to be increased (18, 19), immunoreactivity of hCG was unchanged (15).

Since purification procedures selectively remove partially desialylated, biologically less active molecules of hCG, biologic specific activity of hCG progressively increases to 15,000–20,000 IU/mg. In contrast, for reasons still obscure, immunologic activity plateaus at a level around 5000 IU/mg relative to the Second International Standard hCG (24). As a result, the ratio of immunologic to biologic activity changes progressively with purification. Variable degrees of desialylation arising either prior to secretion or prior to or during excretion partially account for well-known variations in *I/B* ratios of hCG in urine from pregnant women or women with trophoblastic neoplasms (25).

*Subunit structure.*—A second series of observations on the chemistry of gonadotropins has made substantial contributions both to the understanding of discrepancies of *I/B* for human gonadotropins and to understanding immunologic cross-reactions among these hormones. These relate to delineation of the subunit structure of these molecules, now well established for all human glycoprotein hormones as well as for pituitary LH and TSH from other mammals (26–28).

All three human gonadotropic substances consist of two dissimilar subunits, by convention designated α and β, which can be dissociated, isolated, and purified (29–33). Chemical, biologic, and immunologic properties of these subunits have been studied extensively. Recently an amino acid sequence has been proposed for the α (34, 35) and β (34) subunits of hCG. Biologic activities of the subunits have been studied in vivo and in vitro. Specific antisera produced to the α and β subunits of hCG and hLH have been used in neutralization tests and radioimmunoassays to determine the origin of antigenic properties of the human glycoprotein hormones and to compare such properties as plasma half-lives of the dissociated subunits and native hormones. Results of these studies merit more detailed examination.

Preliminary studies carried out with subunits prepared from hCG suggested that they possess significant biologic activity (11, 30). However, an anti-α subunit serum which would bind either intact hCG or the α subunit but not the β subunit was shown to neutralize the biologic activity of hCG β subunit preparations (36). Subsequently, additional purification of the β subunit by gel filtration on Sephadex G-100 resulted in lower biologic activity. The $V_e/V_o$ ratios of hCG and its β subunit are such that hCG elutes from the Sephadex column just prior to and usually overlapping with its β subunit. The observed biologic activity progressively decreased across the protein peak, implying that the significantly higher activity noted on the left half of the peak represented a small but significant contamination with intact hCG (37). Similarly, additional purification reduced the apparent biologic activity of the α subunit, supporting the conclusion that both subunits were essentially devoid of intrinsic biologic activity. Consistent with the lack of biologic activity, no ovarian uptake of tritium-labeled α or β subunits was found in vivo (38) and neither subunit significantly inhibited binding of $^{125}$I-labeled hCG or hLH in vivo (39) or in radioreceptor assays in vitro (40, 41).

Despite the lack of intrinsic biologic activity in the purified isolated subunits of hCG, incubation of complementary subunits in equimolar concentrations resulted in recovery of about 80% of the activity of the parent native hormone preparation (42). Furthermore, considerable biologic activity resulted from incubation of α subunits from different glycoprotein hormones from other species with the complementary subunits of hCG (43). In such experiments, the recovered activity was that of the molecule from which the β subunit was derived, which suggests that this portion of the molecule was responsible for target-organ specificity of glycoprotein hormones.

Lack of in vivo biologic activity in the isolated subunits was associated with a short plasma half-life. The initial half-time of disappearance in plasma of intact immature female rats was 6 min for the hCG α subunit, 11 min for the hCG β subunit, and 141 min for native hCG (38). While it was apparent that recombination provided a longer plasma half-life, this did not seem to be the major factor responsible for the lack of biologic activity of subunits. Rather, it was suggested that conformational changes following recombination, demonstrated by the finding of altered antigenic properties of subunits (see below), are major determinants in recovery of biologic activity (38).

*Immunological properties of gonadotropins.*—Antisera produced by immunizing rabbits with purified α and β hCG subunits have been used effectively in comparative studies of human gonadotropin subunits. Biologic activities of hCG, hLH, hFSH, and hTSH were all neutralized by anti-α hCG serum while anti-β serum only neutralized the activity of hCG (Table 1). Results of these studies and the recombination studies discussed above are consistent with the hypothesis that the well-known immunologic cross-reactivity of human glycoprotein hormones results from common antigenic determinants in their α subunits.

This hypothesis was tested further by studies of the antigenic behavior of human glycoprotein hormones in homologous anti-α and anti-β hCG radioimmunoassays (44). When hCG, hLH, hFSH, and hTSH were radioimmunoassayed in a homologous hCG α system, composed of $^{125}$I-hCG α and a specific hCG α antiserum, the inhibition lines were parallel, indicating antigenic similarity among the preparations. On the other hand, when the same hormones were studied in the homologous hCG β radioimmunoassay system, immunologic dissimilarity between native hCG and hLH became apparent. Further studies have been carried out with the subunits of hLH, hFSH, and hCG in the homologous hCG α and hCG β radioimmunoassay systems (44). The α subunits behaved immunologically like their native hormones in the homologous hCG α system in that both the α subunits and native hormones completely cross-reacted. In contrast, incomplete cross-reactivity relative to hCG β was observed among the β subunits and their native hormones when tested in the homologous hCG β radioimmunoassay system (44).

**TABLE 1.** Capacity of hCG α or β antisera in neutralizing the biologic activity of human glycoprotein hormones

| Hormone | Anti-α | Anti-β |
|---|---|---|
| hCG | +[b] | + |
| hLH | + | –[c] |
| hFSH | + | – |
| hTSH[a] | + | – |

[a] hTSH bioassays performed by Jerome M. Hershman, M.D., Veterans Administration Hospital, Birmingham, Ala.

[b] + = significant decrease in activity of hormone in specific hormonal bioassay.

[c] – = no change in activity of hormone in specific hormonal bioassay.

In view of the foregoing observations, a considerable portion of the cross-reactivity observed among antisera to the human glycoprotein hormones can be attributed to common antigenic determinants in the α subunits. These observations have been confirmed in an independent radioimmunoassay system with antisera to hLH subunits (45). The immunologic basis for between species cross-reactivity was explored with the hLH subunit radioimmunoassay systems using human, rat, ovine, and bovine LH subunits (45). Whereas ovine, bovine, and rat LH $\alpha$ subunits failed to cross-react with hLH $\alpha$ in the homologous hLH $\alpha$ radioimmunoassay system, their $\beta$ subunits completely cross-reacted with hLH $\beta$ in the homologous hLH $\beta$ radioimmunoassay. It would appear then that whereas within species immunologic cross-reactivity resides in the $\alpha$ subunit, between species immunologic cross-reactivity resides in the $\beta$ subunit.

Production of hormone-specific antisera to human gonadotropins has been inefficient in that large numbers of rabbits have been required and the antisera produced were either totally nonspecific (46) or have required absorption to be made specific. For example, Franchimont immunized 45 rabbits to obtain a single antiserum capable of discriminating hCG from hLH (47). In contrast, Vaitukaitis et al showed that each of five rabbits immunized with the $\beta$ subunit of hCG made antiserum that detected antigenic differences in hCG and hLH in buffer solution (48). The use of the hormone-specific $\beta$ subunits of gonadotropins will increase the efficiency with which specific antisera can be produced.

**TABLE 2. Incidence of detectable serum hCG in patients with documented neoplasia**

| Type of Neoplasm | Number of Patients | Number of Positive |
|---|---|---|
| Bronchogenic carcinoma | 15 | 1 |
| Gastrointestinal tract | 143 | 27 |
| Gestational choriocarcinoma | 32 | 32 |
| Hemopoietic | 256 | 7 |
| Lymphoma | 249 | 5 |
| Sarcoma | 36 | 2 |
| Testicular | 94 | 56 |
| Other | 125 | 13 |
| Total | 950 | 143 |

One such antiserum made against the $\beta$ subunit of hCG has been used for developing a radioimmunoassay which specifically and selectively measures hCG in plasma samples containing physiologic levels of hLH (49). Clinically, that assay has been effectively used to follow plasma hCG levels of patients undergoing chemotherapy of gestational trophoblastic disease (49). In fact, the specific hCG assay can detect plasma hCG in those patients when their 24 hr urinary excretions of gonadotropins are at levels observed in normally cycling women (49) as well as when their plasma hLH levels are well within expected luteal and follicular phase concentrations (2, 49).

In addition to studies of patients with tumors expected to secrete hCG, the assay has been used to screen more than 900 serum samples from patients with various types of tumors for evidence of "ectopic" production of hCG (9).

Results of these determinations are summarized in Table 2. The specificity and ease of performance of the assay permit its use on an epidemiologic scale in studies of human tumor biology. Since hCG is physiologically present only during pregnancy, detection of the hormone should suggest the possibility of a neoplasm. Potentially, then, the assay may be of use in screening "high risk" populations for early detection of hCG-producing tumors.

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# NORMAL AND ABNORMAL ALPHA CELL FUNCTION 7080

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In the five decades since its discovery, glucagon has only recently gained general acceptance as a hormone with a potentially important metabolic influence in both health and disease (1). It has now been proposed that the pancreatic alpha cell, the source of the glycogenolytic, gluconeogenic, lipolytic hormone, glucagon, and the juxtaposed insulin-secreting beta cell function together as a coupled bihormonal unit which regulates on a moment-to-moment basis the balance of fuels in a "push-pull" system of opposing hormonal forces (1). Furthermore, it has been established that inappropriately increased alpha cell activity is an integral part of the genetic diabetic state (2–6) and may well adversely influence the disease and its treatment (7). Finally, the role of glucagon in a variety of stressful catabolic illnesses is also receiving increasing consideration (8, 9). This paper will attempt to summarize briefly these aspects of alpha cell physiology and pathophysiology.

## NORMAL ALPHA CELL FUNCTION

Mammals achieve optimal conservation of ingested nutrients through their storage in macromolecular form, e.g. glycogen, protein, and triglycerides. Whenever fuel balance becomes negative, as in starvation, exercise, or disease, these macromolecules can be broken down to their component fuels, glycogen providing glucose for glucose-dependent tissues such as the brain, and triglycerides providing free fatty acids, the most expendable of fuels, and glycerol for general use. When the limited glycogen stores have been exhausted, protein catabolism may, by contributing amino acids as a temporary gluconeogenic precursor, maintain the brain's glucose supply pending its adaptation to the use of ketones as fuel (10). However, unless the use of nitrogenous precursors of glucose is sharply limited and body protein thereby conserved, long term survival during starvation is seriously jeopardized.

During the past year we have presented evidence that the anabolic process of fuel storage and the catabolic process of fuel mobilization may be under the influence of the relative concentrations of insulin (glycogenic, antiglycogenolytic; protein anabolic, antigluconeogenic; and lipogenic, antilipolytic) and glucagon

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(glycogenolytic, gluconeogenic, and possibly lipolytic). An index of the relative concentrations of the two hormones, the insulin:glucagon ratio (I/G), appears to change appropriately within the broad spectrum of need for fuel. As shown in Table 1, the I/G, which in normal fasting subjects averages 3.8, is an extremely low 0.4 in starvation and in exhaustive exercise, circumstances in which need for endogenous fuel mobilization is greatest (11). In starvation, depression of the ratio is the consequence of a fall in insulin coupled with a reciprocal but modest increase in glucagon (12); in exercise the decline in I/G ratio is almost entirely the consequence of a fourfold increase in glucagon (13). At the opposite extreme of the spectrum of need, i.e. when exogenous glucose is abundant, the I/G soars, averaging 25 if the glucose is administered intravenously, and as high as 70 if the glucose is ingested. This rise is a consequence of reciprocally coupled change in the concentrations of the two islet cell hormones (3, 4), a marked increase in insulin, and a modest decline in glucagon. The greater effect of ingested glucose must be attributed to the release of a glucose-responsive enteric signal, possibly secretin, which augments both the positive beta cell response (14) and the negative alpha cell response to hyperglycemia (15).

**TABLE 1. Need for endogenous glucose production vs fasting I/G ratio**

| Experimental Situation | I/G |
|---|---|
| Starvation | 0.4 |
| Exercise | 0.4 |
| Low carbohydrate diet | 1.8 |
| Balanced diet | 3.8 |
| Glucose infusion | 16.0 |

The I/G may also influence the disposition of the most precious of the nutrients, amino acids. As shown in Table 2, a protein meal elicits a near doubling of the I/G in normal well-fed individuals (4, 16) an anabolic response which would favor channeling a substantial portion of the amino acids into new protein synthesis. The rise in the I/G is the consequence of a four- or fivefold increase in insulin coupled with a two- or threefold increase in glucagon. The function of protein-induced hyperglucagonemia is believed to be the prevention of hypoglycemia caused by the aminogenic increase in insulin (17); in normal individuals it is completely abolished if exogenous glucose is provided with the amino acids (4). In starving individuals (Table 2), however, the same protein load will not cause a rise in I/G; on the contrary, the ratio either declines or stays the same (16). This would, predictably, channel an increased proportion of the available amino acids into gluconeogenesis at the expense of protein synthesis, a "catabolic" response.

At the opposite extreme, the ingestion of protein during glucose abundance, e.g. during an intravenous infusion of glucose (4), is associated with an enormously exaggerated increase in I/G (Table 2). As expected, this would virtually abolish gluconeogenesis and result in channeling of virtually all of the amino acids into anabolic protein synthesis. This may explain the well-known protein-sparing action of glucose and the effectiveness of the hyperalimentation regimen introduced by Dudrick and co-workers in treatment of certain catabolic states (18). Table 2 shows that the response of the insulin:glucagon ratio to aminogenic stimulation by protein or arginine varies inversely with the magnitude of need for endogenous glucose, rising maximally when glucose is most abundant, moderately after an overnight fast, remaining unchanged after a period of carbohydrate restriction, and declining in total starvation.

**TABLE 2.** Need for endogenous glucose production vs percent I/G response to protein

| Experimental Situation | Percent Δ I/G |
|---|---|
| Starvation | ↓ 50[a] |
| Low carbohydrate diet | ↑ 6 |
| Balanced diet | ↑ 90 |
| Glucose infusion | ↑ 580 |

[a] Arginine infusion.

## ALPHA CELL FUNCTION IN DIABETES MELLITUS

Disturbed function of alpha cells, as well as of beta cells, appears to be uniformly present in patients with genetic diabetes mellitus. The result is an I/G incapable of rising appropriately when need for endogenous fuel production is abolished by abundance of exogenous nutrients. Thus, an influx of exogenous glucose or amino acids fails to elicit a rise in I/G sufficient for optimal disposition and storage of these fuels in macromolecular form (11), and fuel waste results. The inadequate rise in I/G in diabetic patients is a consequence not only of insufficient or absent insulin secretion but also of an inappropriately high level of glucagon secretion (3, 4). In the basal state, the glucagon level of diabetic patients is at least as high as that of nondiabetics, irrespective of the prevailing glucose concentration (3), and further hyperglycemia resulting from the ingestion or infusion of glucose fails to lower it (4). This alpha cell abnormality, coupled with the defect in insulin secretion, means that the I/G does not rise substantially following a glucose load; thus, the postprandial I/G of the severe diabetic is closer to the I/G of starvation than it is to the ratio observed in nondiabetics after glucose. The response of diabetics to a protein meal is also abnormal (4). In nondiabetics, the alpha cell response to protein ingestion is absent during hyperglycemia, but in diabetics the glycemic autonomy of the alpha cell permits a normal or supernormal glucagon response despite concomitant hyperglycemia (4). Therefore, in the juvenile diabetic, in whom endogenous insulin secretion is absent, the ingestion of protein causes a marked fall in I/G because insulin cannot rise whereas glucagon rises more briskly than normal (4). This starvation-like response of the I/G should result in the channeling of an inappropriately large share of the available amino acids into gluconeogenesis rather than into protein synthesis, which may account for the hyperglycemic effect of amino acids in these patients (3).

The above abnormalities of the alpha cell in diabetes do not seem to be secondary to insulin lack as they are not corrected, at least not promptly, by the intravenous administration of exogenous insulin (19). Moreover, since fasting hyperglucagonemia is present even in extremely well-controlled (3) and chemical (6) diabetics, in whom no absolute insulin deficiency is present, it would seem not to be a direct or indirect consequence of insulin lack. Conceivably it represents a primary inherited defect involving both the beta cell and the alpha cell, which of course shares with the beta cell a common anlage; or perhaps there is a primary defect in the surrounding structures of the islet cells which somewhow renders their responses to fuel abundance inappropriate.

However, there is a fourth abnormality of the alpha cell in diabetes mellitus that is unquestionably the direct consequence of insulin deficiency: the extreme hyperglucagonemia of severe diabetic ketoacidosis (3, 20, 21). This hyperglucagonemia averages more than three times the normal fasting level (21) and recedes with the customary insulin therapy of ketoacidosis. Moreover, it is readily induced in experimental animals by creating insulin deficiency with either antiinsulin serum or alloxan (22).

It is believed that hyperglucagonemia constitutes an unfavorable influence upon the net metabolic state of the diabetic patient, exaggerating the metabolic consequences of insulin lack.

## ALPHA CELL FUNCTION IN STRESSFUL ILLNESS

Hyperglucagonemia has recently been observed in nondiabetic patients with certain stressful illness. In nondiabetic patients with severe and moderately severe infection (8), third degree burns of more than 20% (9), or severe and moderately severe trauma (9), striking hyperglucagonemia, ranging from 3–20 times the normal fasting level, is uniformly present. Whereas in nondiabetics this hyperglucagonemia can be partially or fully compensated for by increased insulin secretion and the I/G thus partially or fully corrected, in the severely diabetic patient such hyperglucagonemia cannot be compensated for and may therefore worsen the diabetic state.

Hyperglucagonemia in stressful illness may well be mediated by adrenergic stimulation that will reduce the ability of even the nondiabetic beta cell to increase insulin secretion and to correct completely the lowered I/G. The impairment in glucose tolerance common in such states and the associated negative nitrogen balance may, at least in part, be attributable to the low I/G. The ameliorating effect of hyperalimentation regimens in such states may well depend on their ability to raise the I/G to an anabolic level.

The role of glucagon in human disease is rapidly unfolding and, while its importance in metabolism remains to be defined, presently available information suggests that it probably plays an important role in normal nutrient homeostasis.

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# EXTRACTION OF INSULIN BY LIVER[1]

7081

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The entire pancreatic output of insulin traverses the liver prior to its entry into the general circulation. Thus hepatic extraction of insulin subserves an important role in regulating peripheral insulin concentrations. Furthermore, the liver is an important site for the degradation of insulin (1) and regulation of hepatic carbohydrate metabolism is quite dependent upon insulin (2). It is not known whether these two processes are related or what fraction of the insulin extracted by the liver is utilized for each. Although increases in peripheral insulin levels have usually been assumed to reflect increased secretion of insulin by the pancreas, Stern et al pointed out the paucity of experimental data to support this assumption (3). In humans they measured the rate of delivery of insulin into the general circulation and emphasized that this was not necessarily equivalent to the total insulin secreted by the pancreas since it did not include that fraction of insulin removed by the liver during a single transhepatic circulation. Wright also emphasized that hepatic extraction of insulin could influence the amount of insulin secreted by the pancreas which reaches the systemic circulation (4). Even measurement of portal vein insulin concentrations may not be representative of pancreatic insulin secretion unless portal vein blood flow is also determined. Increases in insulin concentrations in the portal vein have been associated with commensurate decreases in blood flow so that pancreatic insulin secretion remains unchanged (5).

## MEASUREMENT OF BASAL HEPATIC EXTRACTION OF INSULIN

*Studies using $^{131}$I-insulin.*—Several different techniques have been utilized to measure hepatic extraction of insulin and have provided convincing evidence for such a process. Mortimore et al reported that ~40% of 25 mU/ml insulin presented to the isolated perfused rat liver was removed in a single transhepatic passage (6). $^{131}$I-insulin was used as a marker for the metabolism of insulin. As a control, insulin uptake was measured in the perfused hind limb of the rat and was found to be considerably less. Madison & Kaplan studied hepatic extraction of insulin in anesthetized patients at surgery by injecting $^{131}$I-insulin and inulin simultaneously into the portal vein. Fifteen seconds later a brachial artery blood sample was obtained and the relative difference in the insulin and inulin spaces was attributed to hepatic removal of insulin (7, 8). Their results suggested that approximately 54% (range 41–68%) of the 0.65 U insulin injected was removed by the liver during a single passage. When 1.7 mU $^{131}$I-insulin was injected into the portal vein, 51% was extracted by the liver, while when the same amount of insulin was administered into a peripheral vein, hepatic extraction was only 27% (9). These studies, like those of Mortimore et al (6), utilized trichloracetic acid precipitable radioactivity as a marker for insulin. However, Marshall et al have recently emphasized the hazards of using $^{131}$I-insulin and measurements of trichloracetic acid precipitable radioactivity as an indication of insulin metabolism since $^{131}$I-insulin appeared to be handled differently than immunoassayable insulin in the isolated perfused rat liver (10).

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*Studies using insulin immunoassay.*—Samols & Ryder were the first to evaluate hepatic extraction of insulin utilizing immunoassay rather than $^{131}$I-insulin (11). In patients with portal caval shunts or thrombosis of the portal vein, they measured insulin concentrations in the hepatic vein and a systemic artery. Hepatic blood flow was also estimated, permitting them to calculate insulin flux across the liver. Although this study had the advantage of measurements of hepatic blood flow and insulin by immunoassay, the patients had liver disease and altered hepatic hemodynamics which may have influenced the results. In four such patients, they reported that an average of 41% of the 0.2 mU/kg/min endogenous insulin reaching the liver was extracted by that organ. The range was 19 to 47%. The mean hepatic extraction was similar to that reported by Mortimore (6) and Madison & Kaplan (7, 8). Significantly lower basal hepatic extraction of insulin was suggested by the studies of Waddell & Sussman (12) who used unanesthetized dogs and measured insulin by immunoassay in the portal and a peripheral vein. When the portal vein insulin concentration was below 50 μU/ml, there was not a very large gradient between the portal vein and peripheral vein insulin concentrations, suggesting very little extraction of insulin by the liver. However, in these studies, the absence of appropriate blood flow measurements and hepatic vein insulin concentrations precludes more quantitative estimation of hepatic extraction of insulin. Blackard & Nelson (13) also attempted to estimate basal hepatic extraction of insulin in unanesthetized patients but their results are subject to the same limitations as those of Waddell & Sussman (12) since they did not measure blood flows or hepatic vein insulin concentrations. Under local anesthesia they introduced a catheter into the portal vein via the umbilical vein and measured insulin by immunoassay in this and a peripheral vein. During the control period the portal vein and peripheral vein insulin concentrations were 36.9±4.4 μU/ml and 16±2.9 μU/ml respectively. However, this gradient could be accounted for either by hepatic extraction of insulin or by dilution of the hepatic vein blood by the remainder of the blood volume.

Recently we investigated hepatic extraction of insulin in anesthetized dogs by measuring insulin by immunoassay in the portal vein, an hepatic vein, and the femoral artery.[2] Blood flow was measured by electromagnetic flow meters on the portal vein and hepatic artery. It is important to measure these flows separately since the insulin concentrations in these two vessels may be quite different. Such flow measurements permitted quantitative determination of insulin flux across the liver assuming that the insulin concentration in the one hepatic vein which was sampled was representative of all the hepatic veins. Since the studies of Kanazawa et al indicated that this assumption might be incorrect (14), the experimental procedure was modified in some of the dogs to obtain a more representative sample of mixed hepatic vein blood for insulin determination. In these experiments, a catheter was placed in the inferior vena cava just above the entrance of the hepatic veins and the inferior vena cava was ligated just above the entry of the renal veins. Measurements of blood flow in the inferior vena cava at the site of the inferior vena cava catheter were very similar to the sum of the flows in the portal vein and hepatic artery. The results obtained in dogs prepared this way were quantitatively very similar to those in dogs with a catheter in a single hepatic vein. The mean portal vein insulin concentration in fourteen dogs was ~40 μU/ml, significantly greater than both the hepatic vein or the femoral artery. On the basis of blood flow determinations, ~11 mU/min insulin reached the liver with a range from 3.7 to 41.5 mU/min. During this time 5.3 mU/min insulin was leaving the liver with a range from 1.9 to 13 mU/min. Although the mean extraction of insulin by the liver averaged 43±3%, similar to what had been reported by others (6–8, 11), there was great variability from dog to dog with a range from −20 to as high as 90%. Since these studies were done in anesthetized dogs, they may not be relevant to the true basal state. Thus the results obtained with a variety of techniques and utilizing different experimental models consistently support the concept that in the basal state approximately half of the insulin presented to the liver is removed by that organ during a single transhepatic passage.

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[2] Kaden, M., Field, J. B. 1972. Unpublished observations.

## HEPATIC EXTRACTION OF INSULIN AFTER GLUCOSE

Although there is general agreement about the fraction of insulin extracted by the liver during fasting, disagreement exists concerning the effects of glucose and its concomittant increase in insulin secretion on removal of insulin by the liver. Initially Madison & Unger proposed that a constant percentage of the insulin presented to the liver was removed by that organ (15). Subsequently Kaplan & Madison reported that administration of glucose decreased the fraction of insulin reaching the liver which was removed by that organ during a single transhepatic passage (8). This would permit more insulin to reach the periphery to facilitate the metabolism of the carbohydrate. Infusion of 9 g glucose over 17 min into their patients at surgery reduced hepatic extraction of insulin from an average of 54 to 38%. Only 7.8% of the administered $^{131}$I-insulin was removed by the liver when 52 g glucose was infused over 165 min. Contrary findings were suggested by the studies of Waddell & Sussman mentioned previously (12). Prior to glucose administration, the portal vein insulin concentrations were approximately twice those in the inferior vena cava while after glucose the difference was threefold. In some experiments, after glucose the portal vein insulin concentrations were as much as ten times greater than in the inferior vena cava. When the portal vein insulin concentration was greater than 500 μU/ml, almost all of the insulin appeared to be removed by the liver. Augmented extraction of insulin by the liver is also supported by the observations of Blackard & Nelson (13). Following 25 g glucose injected IV over 2 min in unanesthetized patients, the peak portal vein insulin concentration was about 500 μU/ml. The insulin concentration did not increase proportionately in peripheral veins and the maximum peripheral vein insulin level was achieved after that in the portal vein.

Our results in anesthetized dogs also indicate increased hepatic extraction of insulin following glucose administration.[2] Within 5 min after glucose was introduced into the duodenum, the average portal vein insulin concentration and the total amount of insulin presented to the liver increased significantly without any change in portal vein or hepatic artery blood flow. In some dogs, this change was observed within 1 min after glucose administration and preceded any detectable increase in the femoral artery blood glucose concentration. In seven of the fourteen dogs, the increased insulin reaching the liver was not immediately reflected in higher insulin concentrations in the hepatic vein and femoral artery, indicating augmented hepatic extraction of insulin. Within 1 min after glucose administration, the percentage of insulin presented which was removed rose to 48% from the control value of 43%. At 5 min, when the amount of insulin reaching the liver had increased significantly, hepatic extraction was also significantly greater at 50±7%. Even after the insulin concentrations rose in the hepatic vein, there continued to be increased extraction of insulin by the liver. The peak portal vein insulin concentration in the fourteen dogs ranged from 142 to 1862 μU/ml and was associated with significant increases in femoral artery glucose concentrations. During this time, from 22 to 258 mU/min insulin reached the liver. The average percentage of insulin extracted by the liver increased to a maximum of 69±9% at 40 min after glucose administration. However, after this time the fraction of insulin presented which was extracted by the liver declined toward the control values even though the average amount of insulin presented to the liver continued to increase. Of the fourteen dogs studied, nine demonstrated a significant increase in hepatic extraction of insulin after glucose administration. In these dogs, the maximum insulin which was extracted ranged from 64 to 95% of the insulin reaching the liver and in five of them it exceeded 90%. Despite this very high extraction rate, the arterial insulin concentration still rose reflecting the very marked increase in the absolute amounts of insulin reaching the liver. In five of these dogs, the peak of insulin extraction occurred when the largest amount of insulin reached the liver. The reason that hepatic extraction of insulin was not increased in five of the fourteen dogs is not apparent. The increase in insulin reaching the liver after glucose was similar in these dogs to that in the nine dogs with augmented hepatic extraction of insulin. A possible important factor might be that four of these five dogs had control values for hepatic insulin extraction which were over 60% and two of them exceeded 70%. This is probably not the entire explanation since two of the nine dogs with increased hepatic extraction of insulin had control values of 61 and 80% respectively.

Following the administration of glucose, hepatic glucose balance changed from glucose output to glucose uptake. The amount of insulin reaching the liver correlated significantly with hepatic glucose uptake. However, a consistent relationship between the fraction of the insulin presented to the liver which was removed in a single passage and net hepatic glucose uptake could not be established. The importance of the increased hepatic extraction of insulin after glucose administration is compatible with the observation that a significant fraction of such glucose is metabolized by the liver and that this process is facilitated by insulin (2, 16).

## FACTORS REGULATING HEPATIC EXTRACTION OF INSULIN

*Hyperglycemia.*—Although glucose administration resulted in a greater percentage of the insulin being extracted by the liver, such hyperglycemia is associated with increased insulin secretion. Either or both of the increased glucose and insulin presented to the liver might regulate hepatic extraction of insulin. However, hyperglycemia is unlikely to be the primary signal for increased hepatic extraction of insulin because of our observation that augmented extraction began as soon as there was an increase in the amount of insulin reaching the liver and prior to any significant increase in the amount of glucose presented to that organ.[2] Furthermore, Waddell & Sussman found no relationship between the glucose concentrations of the portal and peripheral veins and the insulin gradient between these two vessels (12).

*Amount of insulin reaching the liver.*—The studies of Kaden & Field indicated that the amount of insulin presented to the liver was an important determinant of hepatic extraction of insulin since after glucose administration there was often increased removal of insulin even before any elevation in the portal vein glucose concentration.[2] Marshall et al also reached similar conclusions (10). Using isolated rat liver perfusion and immunoassay of insulin, they found that hepatic uptake of insulin was directly dependent on the amount of insulin presented. Waddell & Sussman increased the amount of insulin reaching the liver by administering either glucose or tolbutamide to their dogs (12). They observed that the insulin gradient between the portal and peripheral vein was directly related to the portal vein insulin concentration. However, the results of Samols & Ryder in patients with portacaval shunts do not suggest a primary role for insulin in regulating hepatic insulin extraction (11). Infusion of insulin (0.1 U/kg/hr) into a peripheral vein of such patients did not influence the percentage of endogenous insulin extracted by the liver. They were able to distinguish between endogenous and exogenous insulin using relatively specific antibodies. Their results could have been influenced by intrinsic liver disease and altered hepatic hemodynamics which their patients had. The route by which the insulin reaches the liver may also be important in controlling hepatic extraction of insulin.

*Hepatic capacity to extract insulin.*—The results of Madison et al suggest a limited capacity of the liver to extract insulin with saturation of this process at relatively low insulin concentrations (7, 8). Samols & Ryder reported that hepatic insulin extraction followed first-order kinetics and was not saturated when arterial insulin levels were as high as 250 μU/ml (11). Furthermore, the increased extraction of insulin observed after glucose administration and elevation of the amount of insulin reaching the liver (12–14) indicate a very large capacity of the liver to extract insulin. Kaden & Field reported that when more than 32 mU/min insulin reached the liver, consistently more than 60% and frequently over 90%

of it was extracted in a single transhepatic passage.[2] When <32 mU/min insulin was presented to the liver, hepatic extraction was considerably less and sometimes close to zero. Even when as much as 350 mU/min insulin reached the liver, ~80% was extracted. Turner et al (17) and Silvers et al (18) also concluded that the liver had a large capacity to remove insulin. This is substantiated by the studies of Waddell & Sussman (12) since extraction ratios were much higher when the portal vein insulin concentration was 500 μU/ml as compared to when it was 50 μU/ml.

## General Comments

Despite the variability in the amount of insulin presented to the liver and the fraction of it which is removed by that organ during a single transhepatic passage, the absolute amounts of insulin reaching the peripheral circulation are adequate to account for the insulin delivery rates into the peripheral circulation measured by Stern et al (3). In their experiments they made a clear distinction between pancreatic insulin secretion and that amount of insulin reaching the periphery because they were unable to measure insulin removal by the liver. The considerable variation in insulin delivery rates in different subjects at essentially identical blood glucose concentrations could reflect differences in either pancreatic insulin secretion or alterations in hepatic extraction of insulin.

There is very little quantitative information concerning the fate of the insulin extracted by the liver. Although the liver is an important organ for degradation of insulin (1), some of the extracted insulin almost certainly influences hepatic carbohydrate metabolism and is responsible for the reduction in hepatic glucose output. Whether degradation of insulin is an integral part of its physiological action is unknown. Thus, hepatic extraction of insulin is important for the regulation not only of hepatic carbohydrate metabolism but also for the amounts of insulin that reach peripheral tissues and modify metabolism at these sites. Although the details of the control of hepatic insulin extraction are unclear, the amount of insulin presented to the liver seems to be an important factor.

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# PRIMARY HYPERLIPOPROTEINEMIA IN INFANTS 7082

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## INTRODUCTION

Several trends are converging to lend importance to the subject of this review. There is increasing belief that the high incidence of premature atherosclerosis in industrialized societies is not inevitable and that prophylaxis should be designed to retard the formation of arterial plaques rather than to promote their regression. The long lag between morphologic appearance of lesions and clinical manifestations suggests that preventive efforts should begin in children and possibly in infants. Perhaps the most significant of the variables known to be related to atherogenesis is increased plasma concentrations of certain lipids and lipoproteins. These developments enhance the pertinence of information about hyperlipidemia in children and prompt this brief summary of some of the current knowledge about plasma lipids in early childhood. We will place emphasis on cholesterol and low density lipoproteins (LDL), the two factors best correlated with premature atherogenesis, and begin with the neonate.

The principal lipids in plasma are free and esterified cholesterol, phospholipids, triglycerides, and free fatty acids (FFA). All are transported as lipid-protein complexes; FFA are bound to albumin, the others to globulins. Normally more than half of the cholesterol is found in LDL, the next largest share being in high density lipoproteins (HDL). Well over half of the phospholipids are found in HDL. Plasma triglycerides come from two sources: 1. Glycerides in the diet enter the blood, along with sterols and phospholipids, in *chylomicrons*, particles normally present only a few hours after fat ingestion. 2. Glycerides synthesized in the body, from precursors such as FFA, enter plasma mainly from the liver and small intestine in very low density lipoproteins (VLDL). These contain certain of the apolipoproteins common to LDL and HDL. The plasma LDL are at least partially derived from VLDL, and the concentration of HDL is also affected by VLDL turnover and metabolism.

*Normal plasma lipids and lipoproteins in infants and children.*—Fetal cholesterol was first measured in 1912 (1), and scores of reports comparing the plasma concentration of cholesterol (C) and other lipids in maternal and cord blood have appeared since. Those prior to 1955 were summarized by Rafstedt as part of the most comprehensive study of the subject to that time (2). The methods used have varied greatly. The C in human cord blood has been reported to vary from 20 to over 200 mg/100 ml (3, 4). The breadth of this range may be partly methodologic; it extended only from 59 to 94 in 36 control cord bloods obtained under carefully standardized conditions (5). In 37 studies the mean C has varied from 34 to 118 (2–38), but in 24 of these reports it lies between 60 and 79 (2–6, 8–16, 18, 20, 23, 27, 29, 31, 32, 34, 35). In the largest single study of 1800 live births by Glueck et al (3), the mean ±SD was 65±19. The activity of lecithin cholesterol acyltransferase (LCAT) in cord blood has been reported to be in the adult range (8), and the percentage of cholesterol present as esters (2, 6, 11, 16, 27, 31, 34, 38, 39) is comparable to the partition in adults (65–75%). The mean phospholipid concentration lies between 90 and 110 in 10 of 18 reported analyses (2, 10–18, 20, 21, 29, 31, 32, 37, 39). Four of five studies of the triglyceride concentration (5, 14, 16, 18, 25) agree very well at a mean figure of close to 35 mg/100 ml. The range in one study was 18 to 78 (5).

In cord blood the ratio of HDL to LDL is higher than it is later in life. In 36 controls the total C of 74±11 mg/100 ml was distributed in the three lipoprotein families as follows: $C_{VLDL}$ 6±4, $C_{LDL}$ 31±6, and $C_{HDL}$ 37±8 (mean SD) (5). This distribution is compatible with that for lipoproteins measured by electrophoresis (2, 3, 5, 19, 22, 37, 39–41) or analytical ultracentrifugation (42). Fetal HDL are a little over half, LDL about one fifth, and VLDL about one twentieth of their respective concentrations in maternal plasma at term. Differences in ethnic or socioeconomic background, including dissimilar diets, are associated with great differences in maternal lipoprotein levels, yet fetal levels are indistinguishable (3, 6, 9, 15). Such clinical data support other experimental evidence of the relative impenetrability of the placental barrier to the crossing of lipids other than FFA from mother to child (43).

Fetal lipoprotein levels are not totally independent of maternal regulation. Infants of diabetic mothers, for example, have higher cord blood C than infants of normal mothers (10, 12, 44). Females may have significantly higher C than males (4, 9), although sex differences in C, $C_{LDL}$, or $C_{HDL}$ are not always apparent (5). There is also a suggestion that C increases during fetal life. Compared to term infants, C is lower in premature infants and higher in postmature infants (11).

The fetus is nourished continuously on a low fat, low protein, high carbohydrate diet. At parturition it encounters abruptly, and for the first time, a fasted state. Carbohydrate stores are soon exhausted and blood glucose falls. The infant responds by hydrolyzing adipose tissue stores to meet energy requirements. Low concentrations of FFA at birth of .25–.4 mmol/l begin to increase between 1–2 hr of age. Within 4–24 hr they become as high as 1.0 to 1.25 mmole/l (13, 45, 46). There is a parallel increase in plasma glycerol concentration, and ketone levels rise as increased amounts of FFA are utilized for energy (13). The rise in FFA and glycerol can be diminished by feeding glucose from birth (47).

Within the first 10 hr of extrauterine life and before the first feeding the increased flux in FFA manifests in increased VLDL production. The concentrations of (endogenous) triglyceride and VLDL may be doubled (16). During the rest of childhood, they apparently remain about the same. If measured in the postabsorptive state, HDL concentrations rise roughly 25% in the first week of life (2, 19, 40, 48), and then increase very slowly. We have found the mean $C_{HDL}$ to be about 50 (compared to 36 at birth) in apparently normal children between ages 1 and 19 years (49). The mean $C_{HDL}$ in the second decade exceeded that in the first decade by about 10% (5). It has not been established at what age the higher mean HDL concentrations in females first become apparent. The concentration of the major product of VLDL metabolism, LDL, also rises in the first day (13, 40, 50). This impetus to LDL production is further heightened by the onset of feedings, which include the fat and cholesterol in milk.

A mean increase of about twofold in the neonatal C by the end of the first week is due mainly to augmented LDL levels. Normal infants fed either breast milk or the usual cow's milk formulas will have a mean C of 120 to 155 mg/100 ml at one week (2, 4, 13, 31, 35, 51). Thereafter, the rise of plasma cholesterol, now mainly an index of changing LDL concentrations, continues at a slower rate during the first year. From the mean increases observed in three studies (2, 4, 52), it appears that the mean plasma C at 1 month of age in American or British children rises an average of 20% by the end of the first year. In Caucasian children from industrialized cultures C and presumably LDL concentration usually rise another 10 to 15% over the period from age 1 to 10 (15, 52, 53). The available data do not agree upon the inevitability or extent of any rise in the second decade (15, 52–56). In some populations studied girls have had 5–10% higher cholesterol than boys (4, 52, 56–60), but no sex difference has been observed by others (53, 61).

Numerous experiments of relatively short term have been made of the effects of infant nutrition on plasma C. In the first months of life the feeding of human breast milk and cow's milk results in similar plasma cholesterol levels, even though human milk is significantly higher in polyunsaturated fats (7, 62, 63). A formula composed of skim milk supplemented with vegetable oils (such as corn oil) results in a lower plasma cholesterol than does either human breast or cow's milk (62, 64, 65), and a formula consisting of a vegetable protein (such as soya) supplemented with vegetable oils results in even lower levels (14, 62). The available scanty information suggests that diet is a far more powerful governor of plasma lipid levels in infancy and childhood than any genetic factors determined by race. Older children of affluent societies have higher C than children raised in less wealthy cultures (54, 66), and there appears to be a correlation between C and the amount of animal fat in the diet.

Australian and aboriginal New Guinea children offer an interesting contrast in evolution of plasma lipids (15). The mean cord blood C in Australian and New Guinea infants is indistinguishable (70 mg/100 ml), and rises to the same level (150 mg/100 ml) in the first year, during which time the New Guineans are completely breast fed. After the advent of solids in the diet the Australians show a progressive rise in cholesterol through adolescence. As the New Guineans are weaned to their usual diet, which is very high in carbohydrates and low in both fat and protein content, their plasma C declines; by late adolescence the mean is only about 120 compared to over 180 in Australians. These differences, of course, are not necessarily all attributable to diet.

## HYPERLIPOPROTEINEMIA

The dividing line between healthful and unhealthful concentrations of substances in biological fluids is rarely, if ever, sharp. The harmful effects of small excesses may be obscure and will be determined by individual host responses that vary over a wide range. Fiducial limits calculated from the "normal" distribution are therefore often selected arbitrarily. The upper 95% limits for C and $C_{LDL}$ of 270 and 190 in 30–39 year old men (49), for example, are statistically valid but do not represent physiologic limits. They probably should be no higher than 220 and 170 on the basis of demonstrated increase in risk for premature ischemic heart disease (IHD) (67). As calculated from pooled samples obtained from American children 1 to 19 years of age, the upper 95% limits are C 230, $C_{LDL}$ 170, $C_{LVDL}$ 25, and triglycerides 140 mg/100 ml (49). Subpopulations of young American children that are genetically different, i.e. siblings, some of whom have type II hyperlipoproteinemia, are best separated by a C of about 220 and $C_{LDL}$ of about 170 (5). By today's standards any infant with $C>220$ or triglyceride concentration $>140$ clearly has hyperlipidemia.

Lipids and lipoproteins are not commonly measured in infants and relatively little is known about the association of hyperlipidemia with intercurrent illnesses. Probably the most severe secondary hyperlipidemia is seen in congenital biliary atresia (increased unesterified cholesterol, phospholipids, and abnormal lipoproteins), and glucose 6-phosphatase-deficient glycogen storage disease (chylomicronemia, and great increases in VLDL and triglycerides). Primary or genetic hyperlipoproteinemia is known to occur in infants in two forms: type II, believed to have a frequency between 1:100 and 1:1000; and type I, which is quite rare.

*Familial type II (familial hyperbetalipoproteinemia, familial hypercholesterolemia, essential familial hypercholesterolemia).*—None of the present names for this syndrome is ideal because even with clearcut familial involvement, an increase in LDL almost certainly does not define a single disease or mutation at a single locus. The type II lipoprotein pattern in children one year or older is arbitrarily defined by a $C_{LDL}>170$; triglycerides and $C_{VLDL}$ may be normal [type IIa (68)] or increased up to 3–4 times (type IIb). With the possible exception of cord blood there have not been sufficient analyses to define hyperbetalipoproteinemia in infancy; possibly the cutoff limit should be 15 to 25% below that used in children over one year of age, or a $C_{LDL}$ of about 140. The cholesterol concentration does not provide a reliable definition of type II in children below one year of age (4).

The large literature on familial hyperbetalipoproteinemia, which deals with a fairly homogeneous-appearing group of families—and which we may call classical familial type II—has been recently summarized (69). It concerns a disorder in which the type II lipoprotein pattern appears in first degree relatives through successive generations as though determined by a single autosomal gene. The apparent heterozygotes have hyperlipoproteinemia in childhood. As adults they often develop tendinous and subcutaneous tuberous xanthomas and have premature IHD at a rate several times that of normals or relatives without type II (70). Until recent years infants detected with type II usually had two affected parents. Such babies have a $C>300$ and at birth may have yellow-orange cutaneous xanthomas on the buttocks, the creases of the hands, and elsewhere on the limbs. Such "homozygotes" also develop tendon xanthomas in childhood and very early evidence of IHD. Death from myocardial infarction may occur as early as 18 months (69). Their severe atheromatosis also often leads to aortic stenosis. We have followed 12 youthful homozygotes at the NIH and more than a score are reported in the world literature (69). In our registry at the NIH we have 238 children under 20 years of age, each of whom has one parent with type II. In these children, the distribution of $C_{LDL}$ is clearly bimodal with a nadir at about 164. There are 107 children above this value, who are considered to be "heterozygotes" for type II, and 131 below 164, who are considered to be normal (5). The segregation ratio and equal sex distribution is compatible with inheritance as an autosomal dominant. It has been suggested recently that "familial hypercholesterolemia" is determined by polygenic or multifactorial inheritance (71). Most previous analyses (69), including a very recent one (72), have concluded the determination of the classical form of familial hyperbetalipoproteinemia or familial hypercholesterolemia to be monogenic.

Reported examples of primary type II hyperlipoproteinemia diagnosed between birth and one year of age are rare. By extrapolation from other young children and from patients diagnosed at birth, we may draw only some probable inferences about type II in infancy: (*a*) The segregation of normals and abnormals by $C_{LDL}$ is sharper than by C; (*b*) Type II is usually expressed in infancy and should be detectable in this period; (*c*) Clinical manifestations other than simple hyperbetalipoproteinemia are extremely rare in infants with only one abnormal parent (heterozygotes); (*d*) There is considerable variability between the degree of LDL abnormality in the affected parent and affected infant—whether the severity of xanthomatosis and vascular disease in the affected parent predicates the later course in the affected child is unknown; (*e*) The unexplained absolute depression in HDL concentration in familial type II patients is evident from birth; and (*f*) Young children with type II usually, but not invariably (5, 73), have normal glyceride and VLDL concentrations whether their parent has type IIa or IIb hyperlipoproteinemia. The significance of this last point is made uncertain by increasing evidence that familial type II hyperlipoproteinemia, especially that accompanied by hyperglyceridemia, is not one, but several disorders. Goldstein et al have examined families of hyperlipidemic propositi identified after a myocardial infarction (74). Using C and triglyceride concentrations they found what they interpreted as three different kinds of familial hyperlipidemia. One was comparable to the "classical" type II described above and the second was hyperglyceridemia (type IV). They also found families in which C or TG elevations alone, and C plus TG elevations were distributed among relatives in an autosomal dominant mode ("combined hyperlipidemia"). The authors suggested that this abnormality manifested early as hyperglyceridemia and that hypercholesterolemia was less expressed in children.

*Detection of type II at birth.*—Earlier isolated examples of identification of heterozygous type II at birth have been summarized elsewhere (69). In 1971 Glueck and co-workers (3) surveyed 1800 cord bloods in a mixed black and white population. They found a cord blood C greater than 100 in 65 of 1800 neonates, and examined 23 pairs of parents. One parent in each of 11 pairs had type II, as did 3 single parents examined. The estimated frequency of type II was 14/1800 or 0.9%. Darmady, Fosbrooke & Lloyd (4) have recently followed 302 English children. The mean value of cord blood C was 78 ± 23. Of 30 infants with $C > 100$, 5 had a C of $> 240$ at one year; their parents were all normal. The one child who at age one had clearcut familial hypercholesterolemia had a cord blood C of 85. They concluded that cord blood C cannot be used to diagnose type II and that the disorder can probably not be reliably detected in infancy. Following up their earlier suggestion (75) that cord blood $C_{LDL}$ could be better used to separate the affected and nonaffected offspring of a parent with type II, Kwiterovich et al (5) have examined 29 neonates who had one type II parent. Cord blood $C_{LDL} > 43$ mg/100 ml (the mean ± 2 S.D. of control concentrations) was preselected as a discriminant for type II, and 15 of the children exceeded this value at birth. Seventeen have been followed past the first year of life. The 6 of these whose $C_{LDL}$ was below the cutoff at birth remained normal; 10 of 11 considered abnormal at birth were patently so at follow-up. The one "false positive" at birth proved to be the only child fed a diet restricted in saturates and cholesterol throughout infancy. When C was tried as the discriminant in cord blood (mean ± 2 SD of the controls of 94) the cord blood and follow-up diagnoses were widely disparate. They conclude that type II can be detected with reasonable accuracy if LDL is measured in cord blood, although a follow-up at one year—with the infant on the usual diet for the population for at least 3 weeks—is highly desirable for confirmation. $C_{LDL}$ can be estimated accurately from lipid analyses on a 3 ml sample of cord blood without ultracentrifugation (5).

*Treatment of type II in children.*—"Errors" in phenotyping related to suppression of LDL levels by altered infant diet emphasize the potential for beginning treatment of type II from birth. Glueck and co-workers have demonstrated that LDL levels in apparently heterozygous infants with familial type II are sensitive to reduction in intake of the saturated fats and cholesterol in milk (76). The relatively simple expedient of starting feeding with fat-poor milk, perhaps fortified with polyunsaturates, and later elimination of liver and egg yolks in the solid feedings gives considerable promise of being able to retain heterozygous children at near normal levels of LDL throughout infancy. Studies of a few type II infants (76, 77) and of a larger number of normal infants (78) suggest that the early deprivation of cholesterol and milk fats apparently does not impair the ability of the infant to adapt to a diet relatively high in saturates and cholesterol after a few months. If this is so, subsequent adherence to a low cholesterol, low saturated, high polyunsaturated diet should maintain LDL levels in many heterozygous type II children within near normal limits throughout childhood. Possible subtle effects of this type of diet on the development of children have not been excluded. Cholestyramine is effective in heterozygotes and with diet can sometimes sustain C and LDL concentrations in the normal range (79). Although it is now being used in younger patients, cholestyramine or its surgical equivalent, ileal bypass, is still mainly reserved in children for type II homozygotes (79). There has been no use of cholestyramine or other hypolipidemic drugs in infants, and there should be none until knowledge of their possible effects on development and the effectiveness of diet alone can be properly assessed.

*Familial type I (familial lipoprotein lipase deficient hyperchylomicronemia).*— About 20% of nearly 40 clearcut examples of this disorder reported up to 1971 were detected in infancy (31), usually because of eruptive xanthomas, or colic, which calls attention to hepatosplenomegaly or lipemia retinalis. Diagnosis can be made clinically. It consists first of the observation that after standing for 24 hr at 4°C, the plasma develops a heavy cream layer on the top (chylomicrons) over a clear infranate. If the child is fed a special low fat formula (80) for 5 days, the chylomicrons disappear, the triglyceride concentration falls below 500, and C drops to 150 or less. Unrestricted feeding promptly causes chylomicronemia and, if continued, C may reach$>$1000 and triglycerides$>$10,000 mg/100 ml. Familial type I has two known imitators in infancy: diabetic acidosis and glucose 6-phosphatase deficiency. The differential diagnosis usually poses no problem.

The features of familial type I have been recently reviewed (31). Vertical transmission of severe hyperchylomicronemia has never been observed, and it is presumed that familial type I represents homozygosity for a rare mutant allele. Other modes of inheritance such as double heterozygosity for two abnormal alleles have not been excluded. No increase in parental consanguinity has been documented. There also exists no certain biochemical marker for the gene(s) in either carriers or those completely affected. Mutations at different loci may be responsible for the disorder now considered to be a single disease. A present difficulty in assessment of such heterogeneity is due to ambiguities encountered in measurement of the activity of lipoprotein lipase. Deficiency of this enzyme has long been considered to be the basic inherited defect. Although the enzyme acts at tissue sites, its activity is usually assayed indirectly as that momentarily displaced in plasma after injection of heparin. As conventionally assayed, total postheparin lipolytic activity in plasma is not all due to lipoprotein lipase. It includes a hepatic lipase relatively resistant to inhibition by protamine or high NaCl concentrations (81). The protamine-inhibitable activity is very low in familial type I, while the protamine-resistant component is not diminished (82). Tissue lipoprotein lipase activity has been reported to be low in affected members of one kindred (83). The chylomicrons of patients with familial type I contain normal amounts of the apolipoprotein, apoLP-Glu, which activates lipoprotein lipase (84).

More needs to be learned about the catabolism of lipoprotein-bound triglyceride in general, and of chylomicrons in particular, before familial hyperchylomicronemia will be clearly understood. Type I in infants can be managed reasonably well by reduction of fat intake to about 25% of calories. Maintenance of a restricted diet must be lifelong to minimize the otherwise frequent attacks of pancreatitis, which occasionally make this a potentially fatal inborn error of metabolism.

*Voids in current knowledge.*—Types III (85), IV (5, 69, 86), and V (69) hyperlipoproteinemia occur in children, although the prevalence is considered less than in adults (69). Virtually nothing is known, however, of the occurrence of these abnormal lipoprotein patterns in very young children. This information is only a part of knowledge about plasma lipids in infancy that must be acquired for sound development of any pediatric program for prophylaxis against early atherosclerosis. There remains a technological gap in the provision of quantitative measurements of triglycerides and lipoproteins on small blood samples. Existing methods are adequate, however, to solve many outstanding problems, especially if the data are appropriately standardized. Longitudinal studies in children from birth onward are highly desirable. They necessarily require many years to complete, however, and now must take place in a climate of changing attitudes and practice concerning the diet of infants and children. Appropriate comparative data in large samples of children of different ages and living in various cultural and geographic environments are also needed for the setting of more accurate limits of "normal." At the same time, investigators studying families for hyperlipoproteinemia must be prepared to examine additions to kindreds from the time of birth. Special attention is merited by all neonates whose mother or father has type II hyperlipoproteinemia. In these children cord blood should be measured to ascertain the limits of detectability of this treatable syndrome. Just as important is the continuation of careful analysis of the effects of those manipulations of the diet that appear to offer such dramatic control of infantile lipoprotein concentration. Finally, the increasing efficiency with which hyperlipoproteinemia can be treated has compelled pursuit of the all-important question of what influence treatment has on premature IHD (87). Current tests in adults conceivably have started with subjects who were too old at the onset. It is probable that the appropriate trial must begin in childhood. This experiment has not yet begun.

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# CURRENT CONCEPTS IN TREATMENT OF LYMPHOMAS WITH SPECIAL REFERENCE TO HODGKIN'S DISEASE

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Long survival of patients with early stages of Hodgkin's disease, with the probability of true cure in most, and significant improvement in survival rates of those with disseminated disease has been achieved. There is hope that the techniques that have produced advances in Hodgkin's disease can be applied successfully to the other types of lymphoma.

Changes in classification and intensification of therapeutic programs have characterized the field of lymphomas. Most of the activity has centered upon Hodgkin's disease.

## HODGKIN'S DISEASE

Valuable information is contained in the papers presented at the Ann Arbor Symposium (1) and in recent reviews (2–4). The concept that localized extranodal disease has no worse prognosis than is associated with its anatomical stage based upon other involvement is important and may increase the number of stage II patients eligible for curative attempts by 25% (5).

In some centers exploratory laparotomy with splenectomy has become a standard method of staging patients with less than stage IV disease (4, 6–9). Evidence of better tolerance by splenectomized patients has been presented, but intolerance to total nodal radiation has not been a major problem (10). The fear of an increased incidence of serious infections in post-splenectomy children under the age of 15 has been expressed (11, 12). Johnson (13) has argued against routine laparotomy, preferring to treat all or some of the abdominal area of patients in clinical stages I and II. Significantly, several centers radiate the abdomen or use chemotherapy for certain high risk patients despite a negative laparotomy. Peritoneoscopy has been suggested as an alternative to laparotomy since liver invasion is usually associated with dissemination (1). Since prolongation of staging with delay of therapy is often caused by performing a laparotomy, the author's institution deletes preoperative lymphangiograms, isotope scans, gastrointestinal studies, bone marrow biopsies, and pyelograms in patients undergoing laparotomy staging, since clips placed by the surgeon are satisfactory guides for later radiotherapy.[1] Since there is evidence that abdominal radiation, chemotherapy, or both should be given to patients presenting with apparent stage I or II disease regardless of abdominal findings, laparotomy may be less important in the future.

The most common (up to 35%) finding at laparotomy has been occult splenic involvement (4, 6–9). Encouraging data indicates that abnormalities of liver function tests such as alkaline phosphatase, transaminase, bilirubin, and BSP are often not correlated with hepatic invasion by Hodgkin's disease (6, 14). Fortunately, invasion of mesenteric lymph nodes that lie outside the inverted Y-type abdominal port is a rarity in patients presenting with clinical stage I and II (4). Radioisotope scanning with gallium$^{67}$ or indium$^{111}$, which results in a high degree of uptake by lymphomas, has great potential for staging (15).

## RADIOTHERAPY

Tumor doses of 3600–4400 rad appear to result in permanent control of 95% of the Hodgkin's disease tumors irradiated. Total nodal radiation therapy was first applied to stage III with favorable survival, though the results obtained in those with IIIB have not been equal to results in IIIA (16).

This type of therapy with the inclusion of splenic or splenic pedicle ports is now being applied as elective therapy to stage I and II patients. Johnson et al have emphasized the need for extension of fields across the diaphragm, though they recommend that the pelvic and groin areas can be deleted in patients with nodular sclerosis. With this type of therapy, five year survival of 90% has been obtained for all stage I and II patients. In those without symptoms (A-type disease) the five year survival was 98% as compared to 76% in those with symptoms (B-type disease) (17). Abdominal extension has occurred in one third of the patients treated with radiation limited to one side of the diaphragm (17).

Results of the national study chaired by Nickson comparing the efficacy of local and extended field radiation of stage I and II Hodgkin's disease are now becoming available (18). Extended field therapy during most of the study was limited to areas above the diaphragm. There was no difference in the incidence of extranodal extension on the same side of the diaphragm, the number of patients with extension across the diaphragm, or the survival of the two groups. Thirteen percent had relapse across the diaphragm. Fifty-seven percent had nodular sclerosis, which was associated with a much better prognosis than those with mixed cellularity, the other common subtype. In a similar study conducted at Roswell Park Memorial Institute the relapse across the diaphragm with conversion to stage III or IV was identical in the local and extended field therapy groups, as was the survival rate. One third of the patients suffered relapse of disease across the diaphragm (19).

Thus it is apparent that extension of radiation fields to the next adjacent but clinically benign lymph node areas may decrease the number of local extensions, which can be easily treated by local therapy, but is not effective in controlling the incidence of serious extranodal disease or relapse across the diaphragm. If extended fields are to be effective, they must be applied to large areas on both sides of the diaphragm.

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[1] Author's unpublished data.

Fuller et al have recommended total abdominal radiation in selected patients with abdominal Hodgkin's disease (20, 21). Kraut et al (22) have recommended the addition of whole liver irradiation for patients with splenic involvement or for those with stage IV disease in which extranodal involvement is limited to the liver. Since parenchymal lung involvement is more likely to occur in the presence of hilar lymph node involvement, low dose lung radiation is applied experimentally in some centers at the time of primary therapy. A technique for the use of thin lung shields to deliver the intended low dosage throughout the course of mantle therapy has been described, but the long term value of this approach has not been shown (23).

High daily doses of radiation in shortened courses of therapy have given equal or superior results in cord compression and peripheral node tumors (24, 25).

Despite the improvement in tumor control gained by modern radiation equipment, new types of complications from the high depth doses are being recorded. In addition to radiation pneumonia (16, 26), pleural effusions (27) and both acute and chronic pericarditis (28) have been recognized. Hypothyroidism may occur in some of those receiving mantle radiation, and radiation hepatitis following whole liver radiation has been characterized. The major catastrophe of transverse myelitis at the junction of mediastinal and para-aortic ports may result from errors in the placement of the edges of adjoining port areas (29). Maladsorption syndromes may occur after total abdominal radiation. A very small incidence of radiation-induced leukemia has been reported, usually of the myeloid type (30).

Bone marrow depression is a common problem during these large extended treatment programs to both sides of the diaphragm. Total nodal therapy must frequently be interrupted due to low blood counts, but most patients can complete the program with necessary time extensions. Thus, 76% of the patients in the Stanford study were able to complete therapy at full dosage (31). Johnson reported that 88% of patients treated with extended radiotherapy (a portion of these had less than total nodal therapy) were able to finish the treatment (17). The bone marrow is usually completely recovered 5 months following completion of radiation (32).

Although these complications are of considerable importance, the probability of death or serious disability is only 5%, a reasonable price to pay for the routinely good results of long term tumor control obtained by radiation therapy.

## CHEMOTHERAPY

With the availability of vinblastine and procarbazine, each of which has therapeutic activity as great as that of any of the alkylating agents (33), but different modes of activity, longer control of disseminated Hodgkin's disease has become possible. In addition, corticosteroids and vincristine are of significant, though lesser, value and are nearly free of bone marrow toxicity, which is the dose-limiting factor of the other major drugs used for this disease.

Following the evidence of the advantage of combination therapy for treatment of acute lymphocytic leukemia, trials of combinations of active agents have been applied with considerable success in Hodgkin's disease. The MOPP program, employing nitrogen mustard, vincristine, procarbazine, and prednisone in 6 cycles of 2 weeks duration each, has gained widespread use following the demonstration of the long-term favorable results obtained in a group of stage III and IV patients who had received no previous chemotherapy and little or no radiation therapy. This program resulted in a complete response rate of 81%, and the median duration of complete remission following cessation of treatment was 36 months (3, 34). The response rate of this drug combination has been confirmed in cooperative group studies when similar patients are compared. In addition, patients who have relapsed after radiation therapy have the same type of response (23, 35). However, with a spectrum of patients including those with relapse after chemotherapy, complete rates are lower, 63% with a median duration of response of 18 months (35, 36). Age, sex, and the presence or absence of systemic symptoms are also significant factors in responsiveness. A highly favorable prognostic factor in duration of response is the achievement of a complete (as compared to partial) response.[2] There is no advantage conferred by nodular sclerosis to chemotherapeutic response, in marked contrast to radiation therapy programs (3).

The MOPP program was compared to intermittent nitrogen mustard in a randomized trial, with the combination showing markedly superior results (37).

Other types of drug combinations have undergone clinical trials in Hodgkin's disease. In a British study a combination of vinblastine, nitrogen mustard, procarbazine, and prednisone yielded response rates comparable to MOPP (38), as has a recent study with vinblastine, cyclophosphamide, procarbazine, and prednisone (39).

An Acute Leukemia Group B (ALGB) study compared MOPP to a five-drug combination schedule containing vinblastine, chlorambucil, vincristine, procarbazine, and prednisone given in 2 week cycles containing either two or three drugs for a 6 month period. Patients were randomly compared to those who had undergone MOPP therapy as well as a third routine containing the same five drugs of the ALGB combination given in the same total dosage but administered sequentially one at a time (35). While all three routines gave nearly equal response rates, the duration of response was superior with the MOPP routine. However, when analysis was limited to those with little or no past therapy, the duration of response was similar in all three groups. Less important factors in good responders were female sex, stage III disease, lack of systemic symptoms, responsiveness to former therapy, and age under 60 years. Only minor difference in response rates and toxicity favored the ALGB five-drug combination over the five-drug sequentially treated group.

Thus, exposure to a variety of available effective agents during a short period of time and continuation of therapy well into the period of complete response seem to be the important aspects of the recently developed routines.

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[2] Author's unpublished data from Acute Leukemia Group B studies.

In view of the rather high relapse rate following completion of the 6 month intensive trials, programs extending therapy have been studied. This has taken the form of MOPP cycles given half as often or maintenance with single drugs, including vinblastine, chlorambucil, actinomycin D, and methotrexate. In one ALGB routine chlorambucil is supplemented by monthly vincristine and prednisone doses. Preliminary reports from the Southwest Group and the NCI (National Cancer Institute) have shown no difference in remission duration between the groups with or without maintenance (3). However, duration of remission in an ALGB study utilizing maintenance is superior to that obtained in the previous group study, which contained similar 6 month therapy without maintenance (35).

Prolongation of remission after the 6 month intensive course from a median of 24 to 36 months has been achieved by continuation of intermittent cyclophosphamide, vincristine, and prednisone (COP) in the Southwest group (40).

### Survival after Combination Chemotherapy

Despite the advantages of combination drug therapy, relapses are common, inducing the requirement of repeated therapy. While many of the patients retain their tolerance and responsiveness, the rate and duration of response is not as high as in those being treated for the first time. Survival depends as much upon response rates to relapses as well as to the first therapy, and calculations of survival rates are of great importance in assessing the overall contribution of combination chemotherapy. In the ALGB comparison of three routines, MOPP was superior in duration of response, but survival for the three groups was identical. Since nearly all the patients had stage IV disease and none was excluded as being too far advanced for therapy, a survival from this study can be compared to the standard median of between 12 and 24 months for stage IV disease (41–45). Thus, 48+ month median survival of the previously untreated patients indicates a highly significant advance in therapy (Figure 1). Furthermore, the 6 year survival of the entire group from time of diagnosis is better than the usual 3 year expectancy (Figure 2). Figure 2 also indicates that combination therapy can favorably influence survival whether it is the first or later drug therapy used. The NCI group of previously untreated patients has shown a 70% survival rate at five years when only those with complete remission are considered (3). Aisenberg & Goldman recently reported an increase in median survival from 18 to 42 months after institution of chemotherapy, apparently without the benefit of combinations, but likely due to the introduction of vinblastine and procarbazine (46). Thus the obvious improvement in survival is apparently more dependent upon the introduction of new, highly effective agents, and intensification of their use to achieve complete response, than to any one routine of therapy.

### Combined Radiation and Drug Therapy

There are a number of situations in which combinations of radiotherapy and chemotherapy are of value (47, 48), including indications of involvement in organs easily injured by X rays or during initial therapy of stage IV with a dominant tumor mass. Drugs can be used prior to radiation to shrink large tumor masses to

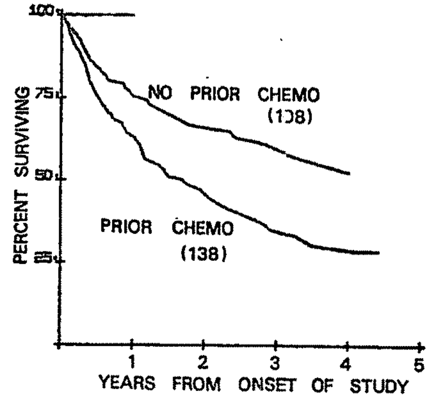

FIGURE 1. The survival in an ALGB study of Hodgkin's disease included 247 patients treated with intensive therapy with four- or five-drug routines for 6 months. The difference in survival between those with or without prior chemotherapy is highly significant.

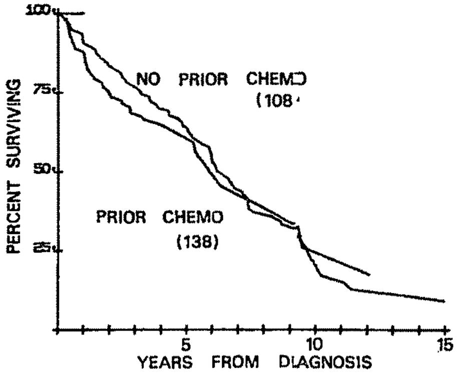

FIGURE 2. In the intensively treated patients of the ALGB study, favorable survival from diagnosis was obtained whether the combination was the first therapy or followed one or more treatments with single agents.

allow smaller radiation ports, to decompress on an emergency basis obstructive syndromes of vena cava, spinal cord, or airways, or to control serious toxic symptoms prior to radiation. Use of three weekly doses of vincristine during radiation has been shown to improve end results of treated tumors without potentiating marrow toxicity (47).

The conjunction of chemotherapy with radiotherapy in stage I and II disease has resulted in productive clinical trials. A randomized British study compared survival of patients, half of whom received nitrogen mustard following radiation, and found significantly improved survival of 88% versus 70% at three years in favor of those receiving the chemotherapy (49). Moxley et al obtained an 86% remission rate in a group of stage I–III patients with combination chemotherapy followed by radiotherapy (50).

In a European cooperative study stage I and II patients received extended field radiation to one side of the diaphragm, followed by vinblastine weekly for 2 years in half the patients. No difference was seen in those with lymphocytic predominance or nodular sclerosis, but those with mixed cellularity and lymphocytic depletion showed significantly better results with chemotherapy: a 4 year survival of 100% as compared to 50% for those treated with radiation alone (51).

A recent report from the Stanford group compared total nodal radiation alone to the same radiation plus 6 cycles of MOPP for stages IB, IIB, IIIA, and IIIB Hodgkin's disease. The number of relapses in the combined therapy group was 1/48 compared to 10/45 in those receiving radiation alone (52).

Thus there is evidence for maximal therapy for at least some high risk types of early stage Hodgkin's disease being achieved by initial combination of radiation and drug therapy. The order of the tandem treatment is not yet clear, but a recent progress report from an NCI study indicates clearly better results with radiation followed by chemotherapy, rather than vice versa (53).

In addition to bone marrow toxicity, other types of early or late toxicity from heavy drug therapy is appearing. The neurotoxicity of vincristine is an important factor in ambulatory outpatient care, and limitation of single doses to 2 mg has been suggested (54). Male sterility, quite likely permanent, was reported in a high proportion of patients treated with MOPP (55). Conversely, normal pregnancies in females after completion of combination therapy have been frequent.[3]

As detailed analyses of large series of patients become available, calculations of risk based upon sex, age, stage, cell type, evidence of vascular invasion on section, and initial responsiveness to therapy may allow the choice of proper therapy balanced against its toxicity for the individual patient (56).

## OTHER LYMPHOMAS

Changes in the classification of these types of lymphoma have been recommended, with the deletion of giant follicular lymphoma category. Instead, the terminology of *diffuse* or *nodular* would be applied to all non-Hodgkin's disease categories. Generally, those with any degree of nodularity respond better to treatment and have better survival. Recommendations for subclassification of the current lymphosarcoma into well or poorly differentiated lymphoma of lymphocytic type, and mixed cell or histiocytic lymphoma for the current reticulum cell sarcoma have been proposed (3, 57).

There is a common tendency to use the anatomical staging system of Hodgkin's disease for these non-Hodgkin's lymphomas, but in view of lack of evidence of uniform sites of onset or orderly manner of progression, such staging is of less value in these types of lymphoma. A high incidence of bone marrow invasion at the time of diagnosis has been reported in patients with apparent local or regional disease (58).

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[3] Author's unpublished ALGB data.

The frequency of extranodal areas as the apparent primary site of disease has been the subject of recent reviews (59–61). A high degree of curability for truly localized extranodal lesions has been reported, whereas those with dissemination from extranodal primaries have been reported to have a worse prognosis than disseminated forms following nodal disease.

The role of laparotomy staging in the non-Hodgkin's lymphomas remains unsettled. The incidence of unexpected abdominal disease has been quite high (62), and this finding, along with the high incidence of early bone marrow invasion, indicates that truly localized disease constitutes only a small portion of the patients with non-Hodgkin's lymphomas.

Truly localized disease does occur, and long survival rates are obtained from radiotherapy (63). In fact, the radiosensitivity of these tumors is very similar to that of Hodgkin's disease (59, 64), and the limitation is in defining the cases with true localization. The value of extended field radiation therapy for these diseases has not been proven, though Johnson has indicated a favorable effect on survival in lymphosarcoma (65).

Drug therapy has been less successful than in Hodgkin's disease. While vincristine gives high response rates in reticulum cell sarcoma, the duration of response, even with continuation of as much as allowed by neurotoxicity, is short and the drug may have only a low order of effectiveness in lymphosarcoma (66).

Alkylating agents are of primary importance in the treatment of these forms of lymphoma (4). Cyclophosphamide is widely used for reticulum cell sarcoma, while nitrogen mustard or chlorambucil is more often used in lymphosarcoma. Bruce has reported data indicating that the various alkylating agents vary considerably in their kinetics of cytotoxicity upon both tumor and marrow stem cells, suggesting that different types of alkylating drugs might be productively combined or used sequentially (67). Recent data from clinical studies with BCNU would tend to confirm this lack of cross-resistance, and CCNU may be an even more effective nitrosourea compound (68, 69).

Combinations of drugs have received wide attention in the non-Hodgkin's lymphomas. A mixture of cyclophosphamide, vincristine, and prednisone has undergone intensive cooperative group trials and was shown effective and superior to cyclophosphamide alone (70, 71). At the NCI these drugs on a somewhat different schedule resulted in as high a response rate in those processes of extranodal origin as in those originating in the nodal form (72). A recent report from Yale indicates a high degree of responsiveness with initial cyclophosphamide and vincristine induction followed by eight weekly doses of cytosine arabinoside and methotrexate with leucovorin rescue (73).

Bleomycin, a new drug with little or no bone marrow toxicity, has shown considerable activity in Hodgkin's disease, but probably much less in other lymphomas. Its pulmonary toxicity is a severe limiting factor (74, 75).

Adriamycin, a derivative of daunorubicin, has shown a high initial response rate against reticulum cell sarcoma, but in addition to severe marrow toxicity, fatal cardiac toxicity may appear after prolonged use (76).

Although non-Hodgkin's lymphomas are highly treatable, improvement in long term results from utilization of recently developed techniques has not equalled that of Hodgkin's disease. The search for further improvements in radiotherapy, chemotherapy, and immunotherapy continues.

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7084

# NATURE AND BIOLOGICAL ACTIVITIES OF DEGRADATION PRODUCTS OF FIBRINOGEN AND FIBRIN

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The recognition of the importance of fibrinogen proteolysis in a wide variety of pathological conditions ranging from disseminated intravascular coagulation to local fibrin deposition in renal diseases has led to numerous studies of the nature of the fibrinogen degradation products (FDP). This review will deal primarily with the biological and clinical aspects of the problem. Details of the chemical and physical characterization may be obtained elsewhere (1–6).

## STRUCTURAL CONSIDERATIONS

Mammalian fibrinogen is made of three peptide chains, $\alpha$ (A), $\beta$ (B), and $\gamma$ (7). The molecule is considered to be a dimeric structure with the formula $[\alpha(A), \beta(B), \gamma]_2$, having a molecular weight of 340,000. The N-terminal ends of the three chains are linked together in a firm disulfide knot (8). Because of the dimeric structure, two N-terminal disulfide knots are located at opposite ends of the molecule, perpendicular to the axis of symmetry (Figure 1). This symmetrical model for fibrinogen bears a close similarity to the divalent symmetrical model proposed for immunoglobulins. The two fibrinogen monomers of 170,000 unit weight are joined by additional "intra-dimer" disulfide bonds, located between the respective $\alpha$(A) and $\gamma$ chains. The regions of biological activity for both types of molecules are believed to reside at opposite poles of the molecule. Thus, al-

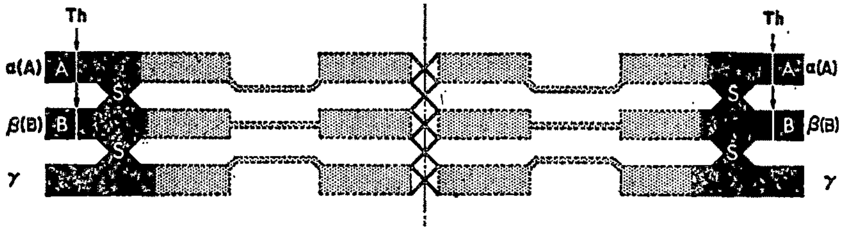

FIGURE 1. Blomback's concept of the fibrinogen molecule. The three chains are denoted as $\alpha$(A), $\beta$(B) and $\gamma$, the fibrinopeptides as A and B and the disulfide knots as S.

though the disulfide knot constitutes only 16% of the whole fibrinogen molecule, it is a very important part containing the reactive parts of the molecule, namely the fibrinopeptides A and B.

During the conversion of fibrinogen to fibrin by thrombin, the fibrinopeptide A is cleaved off the $\alpha$ chain and the fibrinopeptide B is split off the $\beta$ chain. The release of fibrinopeptide A occurs more rapidly than that of peptide B. This proteolytic action of thrombin is highly specific and is limited to the arginyl-glycyl bonds linking these two fibrinopeptides to the rest of the molecule. The chain remains unchanged. The release of negative charges after the breakage of these peptides induces a conformational change in the molecule, opening sites for polymerization of the molecule into fibrin. In addition to thrombin, fibrinogen may also be clotted by trypsin, papain, ficin, and snake venoms (9–11). Two of the latter have been refined; they are Ancrod (also known as Arvin) from venom of *Agkistrodon rhodostoma*, and Reptilase (also known as Defibrase) from that of *Bothrops atrox*. They differ from thrombin by removing only fibrinopeptide A from fibrinogen. The resulting fibrin polymer is structurally different with end-to-end instead of both end-to-end and side-to-side polymerization (12, 13). An interesting enzyme has been isolated from the venom of another viper, *Agkistrodon contortrix* (14), which hydrolyses peptide B faster than peptide A. In this case no clotting occurs until sufficient peptide A is removed, showing the importance of peptide A in the fibrinogen-fibrin conversion.

Hydrolysis of the fibrinogen molecule at other locations may also be brought about by other proteases. However, this review will be limited to the physiological proteolysis by plasmin with the formation of peptide fragments known as fibrinogen degradation products (FDP). There are 160–170 peptide bonds susceptible to the action of plasmin, in contrast to more extensive proteolysis involving approximately 360 bonds by trypsin (15). Unlike trypsin, the fibrinopeptides are not split off by plasmin (16). Thus, after plasmin proteolysis, the fibrinopeptides may be retained in the fragments. These can be further hydrolyzed by thrombin and result in further clotting of the larger fragments.

During the digestion of fibrinogen by plasmin, several stages of fragmentation of the molecule have been identified. In the earliest stage of digestion, a large molecular species is formed termed fragment X (mol wt 240,000) (17), also known as first intermediate product (3), first fibrinogen derivative (5), fractions I-8 (18) and I-8$D_5$ (19). This is clottable by thrombin, though at a much slower rate than by fibrinogen.

During the next phase of digestion, two intermediate degradation products, fragments X and Y, are found. These were also termed by earlier investigators as intermediate fibrinogen derivatives (5), and the fragment Y was termed the second intermediate product (3). The "final" fibrinogen degradation products reduced after prolonged digestion were identified by DEAE-cellulose column chromatography and electrophoretic mobility in agar gel as five products labeled "fragments A, B, C, D, and E" (20). The molecular size of the largest of these "final" products, fragment D, is 80,000. Since the rate of digestion varies according to the conditions, digests of identical incubation times may differ considerably in

**TABLE 1. The physical characteristics of fibrinogen, the various FDPs, and the complexes**

| | Fibrinogen | X | Y | D | E | ABC | Fibrin Monomer | Complex Polymer | Fibrin Monomer and FDP |
|---|---|---|---|---|---|---|---|---|---|
| Molecular Weight | 340,000 | 240,000 | 155,000 | 83,000 | 50,000 | — | 330,000 | 670,000 | 400,000–1,000,000 |
| $S_{20,w}$ | 7.4 | 7.0 | 5.7 | 4.9 | 3.1 | 1.5–2.2 | 7.4 | 9.2 | 11–13 |
| $D_{20,w}$ | 2.1 | 2.6 | 3.2 | 5.1 | 5.0 | — | 2.1 | — | — |
| Elution Volume with Sephadex G-200 ($V_e/V_o$) | 1.0 | 1.04 | 1.23 | 1.61 | 1.61 | 2.0 | 1.0 | 1.0 | 1.0 |

their content of FDPs and must be analyzed each time. The degradation process is generally recognized to be one with the loss of approximately 20% of fibrinogen to produce minor fragments A, B, and C. It is postulated that during the various stages of degradation fragment X (mol wt 240,000) may be split to one fragment Y (mol wt 155,000) and one fragment D (mol wt 83,000). Later, fragment Y may be split to form a second fragment D and one fragment E (mol wt 50,000). Recent studies (21) indicate that fragment E is located at the N-terminal of the molecule and contains the fibrinopeptide A. Thus, the fragment E derived from fibrinogen would be different from that derived from fibrin. Fragment D is located at the COOH-terminal and therefore has the same structure whether it is derived from fibrin or fibrinogen. This fragment also contains the site for crosslinking. Some of the physical characteristics of the fibrinogen degradation products are shown in Table 1.

In addition, these FDPs may form soluble complexes with the parent fibrinogen molecule as well as with the fibrin monomer (22, 23). The complexes range in size from a dimeric structure of fibrinogen-fibrin monomer complex to even larger complexes of fibrinogen with one or more of the FDPs. The molecular weights are estimated to range from 400,000 to 1,000,000. The clinical implications of these complexes will be discussed later.

## CHARACTERIZATION AND PURIFICATION

Most reports on the characteristics of the FDPs are based on two parameters: the molecular size (and weight) and the ionic charge. The use of the analytical ultracentrifuge with determination of the sedimentation coefficient ($S_{20,w}$) provides adequate means for following the digestion products at the different stages. Some useful values related to the molecular size of the FDP and their complexes are listed in Table 1. Further identification can be achieved by gel electrophoresis in either agar or polyacrylamide gels. This can be combined with immunological techniques.

For the analysis of the $\alpha$, $\beta$, and $\gamma$ chains, the sodium dodecyl sulfate (SDS)-urea polyacrylamide gel electrophoresis is most useful. Following the extension of the fibrinogen (or FDP) molecule by urea, the highly charged SDS dissociates it into the three chains. Subsequent separation would be dependent on the size rather than the ionic charge of the fragments (24).

The methods for characterizing FDPs can also be used for their purification. However, those based on molecular size are preferable for large scale preparation. The best of these is gel permeation chromatography using Sephadex G-200. The fibrinogen molecule elutes with the void volume, followed by the peaks X, Y, D, and E, and ABC—eluting in that order. The elution coefficient of the FDPs, defined as the elution volume for the peak $V_e$ divided by the void volume ($V_o$) for the column, $V_e/V_o$, is shown in Table 1. If one is interested in the isolation of intermediate products, X and/or Y, it is necessary to stop the digestion with plasmin by the use of known plasmin inhibitors such as soybean trypsin inhibitor. Another possible way to stop the reaction would be the attachment of the plasmin to an insoluble matrix which would allow the reaction to be terminated by spinning out the precipitated enzyme. Further separation of D from E can be achieved by passage through a second column such as Sephadex G-150 or G-100 which will allow the separation of fragments with lower molecular weights; alternatively, based on the ionic charge differences, they may be separated by DEAE-cellulose chromatography. Again, the appropriate digestion times for the different preparations must be established in each laboratory due to the variability of the quality and quantity of fibrinogen and plasmin used.

## BIOLOGICAL PROPERTIES

The most pronounced biological effect of FDP is its anticoagulant activity. This may be achieved through different mechanisms. It competitively inhibits the action of thrombin on the fibrinogen-fibrin conversion (23, 26). This antithrombin action (also known as antithrombin VI effect) is strongest with the earlier and intermediate products (fragments X and Y) and is relatively weak with fragments D and E (3). Soluble complexes may also be formed between fragments X, Y, or D and the fibrin monomer, thereby preventing the polymerization of the monomers (3, 23). In addition, FDP may interfere with thromboplastin generation (25). Electron microscopic observations revealed that the structure of fibrin formed in the presence of FDP is faulty presumably as a result of abnormal polymerization (27).

Another biological effect is that on platelet function (26, 28). While the smaller and dialyzable FDPs have been found to inhibit platelet adhesiveness, aggregation, and release reactions (28), the larger FDP and the FDP-fibrin-fibrinogen complexes may have the opposite effect. Thus, the resulting mixed effects may not be clinically apparent when a mixture of the various FDPs is present, such as in the case of disseminated intravascular coagulation. On the other hand, when only the smaller fragments predominate, such as during Ancrod therapy, a distinct impairment of platelet function can be demonstrated (13, 29).

The dialyzable low molecular weight fragments may also cause smooth muscle contraction (30, 31) and enhance the contractile action of bradykinin (31, 32). They may also increase capillary permeability (33) and induce chemotaxis of granulocytes. Thus, in addition to their effects on hemostasis, the FDP may have a role in inflammatory lesions and in the repair of damaged tissues.

## METHODS OF DETECTION

For the purpose of clinical diagnosis, either plasma or serum samples are used. Care must be taken to prevent artifactual production of FDP in vitro after collection of the samples, such as by blood plasmin activity. The usual practice is to add an inhibitor of fibrinolysis such as soybean trypsin inhibitor or epsilon aminocaproic acid immediately upon collection. Obviously immunologic techniques will not be suitable for the estimation of FDP in plasma since the fibrinogen present in plasma will interfere with the results. On the other hand, because the larger peptides such as fragment X and the larger complexes are thrombin-clottable and are thus removed from plasma during the clotting process, serum does not contain the whole spectrum of FDP. Only fragments Y, D, and E and the smaller peptides are present.

The immunologic methods are perhaps the most sensitive of the different detection techniques. Antifibrinogen serum will react with fibrinogen and all its major degradation products. Attempts are being made to isolate fragment-specific antibodies with some success. Antifragment D and antifragment E sera have been produced and found not to cross-react with each other nor with antifibrinogen serum. The specific antisera are useful in distinguishing the different forms of FDPs. Most people, however, rely on immunoelectrophoretic methods for the individual identification of the fragments (2, 4, 5, 17). According to Marder et al (34), the tanned red cell hemagglutination inhibition immunoassay (TRCHII) (35) can detect as little as 0.6 μg per ml of fragment X or Y, or twice the concentration of fragment D. However, fragment E is relatively insensitive to this method of detection (Table 2). For fragment E assay at lower concentrations, the immunodiffusion method of Ouchterlony is used instead. Methods based on

**TABLE 2. Comparative sensitivity of fibrinogen and the various FDPs to the commonly used methods of detection**[a]

| | Fibrinogen | X | Y | D | E |
|---|---|---|---|---|---|
| Anticoagulation assay | − | +++ | +++ | ++ | + |
| Thrombin clottability | +++ | +++ | ± | − | − |
| Staphylococcal clumping | +++ | ++ | ++ | − | − |
| Immunodiffusion | ++ | ++ | ++ | ++ | ++ |
| Flocculation | ++ | ++ | ++ | ++ | ++ |
| TRCHII | +++ | +++ | ++ | + | + |

[a] Levels of sensitivity are indicated by +++ for most sensitive; +, least sensitive; and −, insensitive.

direct observation of antigen-antibody reaction such as the commercial latex "Thrombo-Wellcotest" and the flocculation test (36) are best suited for emergency clinical situations.

Because FDPs have anticoagulant activity, due to an antithrombin action and to an antipolymerization activity, the thrombin time (37) has long been used as an indicator of FDP activity. Its usefulness however, is limited due to the relative insensitivity of the test—particularly for the detection of fragment E. The antipolymerization action is directed against the fibrin monomers formed not only by the action of thrombin, but also by that of Ancrod or Reptilase. Thus, in addition to thrombin time, the Ancrod time or the Reptilase time (38) are also prolonged in the presence of FPD and may instead be used as diagnostic methods. Since heparin interferes with the action of thrombin, the thrombin time cannot be used for the detection of FDPs in clinical situations when the patient is heparinized, e.g. during treatment for disseminated intravascular coagulation. Ancrod and Reptilase, on the other hand, are not affected by heparin and should be used instead.

Another test that enjoyed brief popularity was the staphylococcal clumping test based on the principle that fibrinogen and fragments X and Y have the ability to cause clumping of a suspension of the Newman $D_2$ strain of *Staphylococcus aureus* (39). However, it is relatively insensitive to fragments D and E, and is thus not able to give strong positive results for FDPs in samples from patients who have predominantly small fragments in their blood, such as those occurring after Ancrod or Reptilase treatment.

An interesting phenomenon of gel- or fibrin-strand formation caused by ethanol or protamine sulfate acting on fibrin monomer or large FDP complexes containing fragment X is termed paracoagulation (40). This forms the basis of the ethanol gel (41) and the protamine sulfate paracoagulation tests (40). In primary fibrinolytic disorders, the FDPs are the products of fibrinogen digestion and thus the fibrinopeptides are still attached to the fragments (e.g. fragment $X^{fp}$). These fragments do not paracoagulate. In contrast, the FDPs in disseminated intravascular coagulation are formed from the breakdown of fibrin and are devoid of the fibrinopeptides (e.g. fragment $X^{o}$). Paracoagulation will take place. Furthermore, when intravascular coagulation occurs, there is thrombin action on the fibrinogen and thus fibrin monomers will be present. They, too, give the positive paracoagulation results. The ethanol gelation test is relatively insensitive and frequently gives negative results in cases of disseminated intravascular coagulation, while the protamine sulfate test is reported to be quite reliable.

## Clinical Significance

Both the degradation products derived from fibrinogen or fibrin may be found in serum or plasma as a result of either intravascular or extravascular proteolysis of fibrinogen or fibrin. Some of these FDPs may be excreted through the urine where they are not to be confused with FDPs derived from local lysis of renal intraglomerular fibrin deposits or from urinary proteolysis of fibrinogen lost in urine.

Because the therapeutic approaches to primary fibrinolytic disorders and to pathological fibrinolysis secondary to disseminated intravascular coagulation are quite different, many attempts have been made to distinguish the two syndromes by means of the identification of plasma FDP. In primary fibrinolysis, most FDPs are derived from the plasmin digestion of fibrinogen. Thus, the amount formed will be in proportion to the increase of fibrinolytic activity. On the other hand, in disseminated intravascular coagulation the FDPs will be in excess of the level of plasma fibrinolytic activity. Mention has been made in an earlier section of the use of the paracoagulation phenomenon to differentiate fibrin degradation products from those derived from fibrinogen (40, 41). In clinical practice, however, the plasma levels of these products are often too low to be detected by the paracoagulation method. Thus, attempts to distinguish these two types of FDPs are often unsuccessful. Reliance must be placed, therefore, on the associated clinical and laboratory findings and on the relative degree of fibrinolytic activity and quantity of FDP.

The presence of FDP in plasma will also reflect the presence of other forms of intravascular coagulation such as pulmonary embolism (42). The plasma level in this condition is not as high as in the case of disseminated intravascular coagulation. In addition, the extravascular breakdown of fibrin provides another source of serum FDP. Elevated serum FDP levels can thus be found in varying quantities in patients after major operations, and in those having hematomas, malignant tumors, and sepsis (42).

FDP may also be seen in other forms of localized lesions associated with fibrin deposition, for example, those extensively studied forms of renal disease in which intraglomerular fibrin deposition is the characteristic feature, such as in all types of glomerulonephritis (43), particularly in the proliferative form (44), in experimentally-induced renal disease (45), and in renal homograft rejection (46). Plasminogen activator present in both normal and diseased glomeruli is believed to be responsible for the lysis of such fibrin producing the FDP (45, 47). Much of the FDPs are excreted in the urine, so that daily measurements of urinary FDP concentration may be of value in assessing the severity and progress of the renal lesions. An additional source of urinary FDP in glomerulonephritis is the proteolysis of fibrinogen lost in the urine. In nephrotic syndrome where large amounts of fibrinogen may be lost along with other plasma proteins, the identification of the different fragments of FDPs may be helpful in showing the selectivity of the protein loss and thus the prognosis.

The work of Fletcher et al (22) in the quantification of specific fibrinogen-fibrin and FDP complexes opens a new approach to the diagnosis of thrombosis and of the "hypercoagulable state." They reported that while in the normal subject plasma fibrinogen appears as a single molecular species of mol wt 330,000, the plasma of patients with intravascular coagulation, including those in a hypercoagulable state, may contain soluble large molecular weight fibrinogen-fibrin complexes. These are formed from various combinations of fibrinogen, fibrin monomer, and the larger FDP fragments. Their molecular weights range from 400,000 to 1,000,000 with an average around 450,000. They also observed during active fibrinolytic states, such as thrombolytic therapy with urokinase, fibrinogen derivatives of mol wt 257,000 or less in the plasma. These correspond to the FDP derived from the plasmin proteolysis of fibrin. By means of agarose-gel exclusion chromatography using Biogel-5M, they were able to identify the fibrinogen pattern in the plasma samples of a large series of patients with venous thrombosis, hypercoagulability, and active fibrinolysis. If confirmed, this new approach may serve as a useful tool for the identification of these disease states.

## CONCLUSION

The role of fibrinogen beyond its provision of a hemostatic barrier has recently been recognized. Fibrin deposition is an important component not only of a thrombus but also of a wide variety of pathological processes. Studies of the products of fibrinogen and fibrin catabolism have improved the diagnostic approaches to these conditions and contributed to a new understanding of these diseases.

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# CYTOKINETICS IN DISEASES OF EPIDERMAL HYPERPLASIA[1]

7085

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During recent years significant advances have been made in understanding the kinetics and the biochemistry of cell proliferation. Most of the pioneer work has involved the use of experimental animal tumors; it has only been in the past few years that suitable technics have been developed for human tissues to provide a valuable view of the pathophysiology and therapy of certain benign and malignant skin disorders.

## Principles and Methods of Studying Epithelial Cytokinetics

In this section we review both the basic principles of cell proliferation kinetics and the technics that have been developed to study cytokinetics in human skin. Consideration of cytokinetics must be divided into the component parts of the epidermis, i.e. the proliferative cell compartment, and the viable and nonviable (stratum corneum) differentiated cell compartments.

The cell cycle constitutes the time period separating completion of mitosis from completion of the following mitosis in a daughter cell. The reproductive (proliferative, germinative) cycle, critically reviewed by Baserga (1), is characterized by the following four presently known phases as illustrated in Figure 1: $S$, the period of active nuclear DNA synthesis; $G_2$, the period from the end of DNA synthesis to the beginning of mitosis; $M$, the period of mitosis; and $G_1$, the interval from completion of mitosis to the initiation of DNA synthesis. During the $G_1$ phase the individual epidermal cell in the basal layer(s) may either leave the proliferative cell compartment to differentiate by moving outward through the epidermis or remain to repeat the germinative cycle.

The most widely used technic for determining the duration of the cell cycle and its component parts is the labeled mitoses method of Quastler & Sherman (2). This method has been used to study cytokinetics of normal skin and certain diseases of epidermal hyperplasia in vivo (3–6). The experimental procedure consists of injecting small quantities of tritiated thymidine intradermally or intralesionally (instead of large systemic doses) into multiple sites of normal or diseased human skin and obtaining biopsies of these sites at time intervals (3, 7, 8).

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Autoradiographs are then analyzed to obtain a classical labeled mitosis curve from which the duration of the cell cycle and its component parts can be determined.

Similar technics can be used to determine the epidermal cell's average turnover or transit time through the viable differentiated cell compartments, that is, the time it takes labeled cells to proceed to the uppermost stratum granulosum cell layer after leaving the basal layer (5, 6, 9). Isotopically labeled amino acids incorporated into cytoplasmic proteins have been used to measure the transit times through both viable and nonviable differentiated cell compartments (10–12). In this manner the cytokinetics of all three cell compartments in epidermis (proliferative, viable differentiated, and the nonviable stratum corneum) can be measured.

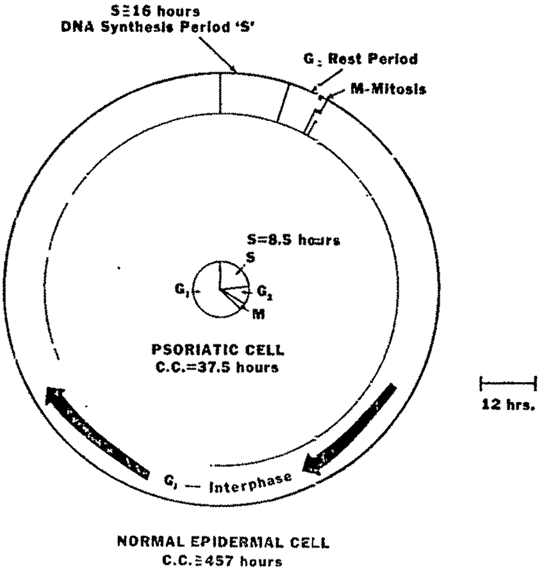

FIGURE 1. Germinative cell cycle of normal and psoriatic epidermal cell drawn to scale to show relative size and duration of each phase. (Taken from *Arch. Dermatol.* 1971. 103:33–38. "Copyright 1971, Am. Med. Assoc.")

The technics described above are all in vivo methods of measuring a dynamic active process over a period of time. Unfortunately, in vitro cytokinetic experiments are limited to a single point in time since adequate organ or tissue culture technics capable of maintaining kinetic patterns are not yet available. In vitro technics have been used to study thymidine labeling patterns in normal skin and premalignant and malignant lesions (13, 14)

## CYTOKINETICS IN NORMAL SKIN AND BENIGN DERMATOSES

The first application of radioisotopes to the cytokinetics of human epidermis was by Rothberg et al (11) who studied turnover time of the epidermis in normal and psoriatic skin. Although the experimental design and technics were relatively crude, their results were a harbinger of further studies. Their data indicated a turnover time of normal epidermis to be approximately 28 days whereas in psoriasis the turnover time was 3–4 days, approximately 7–8 times faster. A similar relationship was found by Van Scott using mitotic and cell counts in normal and psoriatic skin (14). The definition of turnover time versus transit time is now being examined more critically to obtain finite values for epidermal cell proliferation kinetics (15, 16).

In 1965 the local intradermal injection of isotopic compounds was introduced as a technic to study cutaneous kinetics (10). In two simultaneous publications (5, 9) data were presented on the incorporation of tritiated thymidine into the DNA-synthesizing basal cells of human normal and/or psoriatic epidermis. Weinstein & Van Scott followed the movement of these labeled cells through the differentiated viable epidermis to obtain transit times of 12–14 days in normal and 2 days in psoriatic epidermis (9). What determines when an individual cell is ready to leave the proliferative basal compartment for differentiation is still unknown. A cellular kinetic equilibrium in the skin can be maintained only if during each division an average of one daughter cell differentiates and moves outward while the other daughter cell remains in the proliferative compartment (17).

In examining the cytokinetics of normal epidermis Epstein & Maibach found that about 5% of the basal cells incorporated thymidine-$^3$H (5). Using estimations of DNA synthesis times from animal data of 6–8 hr, they calculated the turnover of the proliferative basal cells to take 6 days. Newer data on the duration of the *S* phase in human epidermis upgrades these values. Their measurement of transit time in normal epidermis showed a value of 10–14 days, similar to Weinstein & Van Scott's figures (9).

The turnover of the stratum corneum compartment reflects not only the rate of cell proliferation in the basal layers but also the absolute thickness of stratum corneum. The transit time of psoriatic stratum corneum was measured autoradiographically as 2 days (9). The best measurements of normal stratum corneum transit times are obtained from the fluorescent technic of Baker & Kligman (18). Since the fluorescent compound tetrachlorasalicylanilide penetrates to the base of the stratum corneum, disappearance of fluorescence at 10–19 days gives a range of normal stratum corneum turnover time.

Another variation of intradermal isotope injections for kinetics involves the use of labeled methionine and glycine with collection of scales by cellophane-tape strippings (19). The results confirm the relative differences in turnover times between normal and psoriatic epidermis, but this technic has unresolved difficulties based on the different levels of individual amino acid incorporation in epidermis (20).

**TABLE 1. Comparative in vivo data on cytokinetics in human epithelial tissues**

| Tissue | $T_s$(hr)[a] | LI(%)[b] | $T_{cc}$(hr)[c] | Transit Time[d] | Ref. |
|---|---|---|---|---|---|
| Normal epidermis | 16 | 3.5 | 457 | 12–14 | 9,21 |
| Normal epidermis | – | 5.0 | – | 10–14 | 5 |
| Psoriasis | 8.5 | 22.7 | 37.5 | 2 | 3,9 |
| Ichthyosis vulgaris | – | – | – | 10–14 | 6 |
| Sex-linked ichthyosis | – | – | – | 14–16 | 6 |
| Lamellar ichthyosis | – | – | – | 4–5 | 6 |
| Epidermolytic hyperkeratosis | – | – | – | 4 | 6 |
| Basal cell carcinoma | 19 | 5–9 | 72–96 | – | 25 |
| Basal cell carcinoma | 20 | 9.2 | 217 | – | 26 |
| Malignant melanoma | 24 | 14 | – | – | 27 |
| Malignant melanoma | 21 | 8 | 72 | – | 28 |
| Normal cervix and vagina | | | | | |
| Normal ovarian function | 9 | 14 | 63 | 4 | 6 |
| Postmenopausal unstimulated | 9 | 10.5 | 86 | 4 | 4 |
| Postmenopausal with estrogen stimulation | 9 | 22 | 41 | 4 | 4 |
| Invasive squamous cell carcinoma, cervix | 10.4 | 33 | 15 | – | 29 |
| Invasive squamous cell carcinoma, cervix | 8 | 25 | 31 | – | 30 |
| In situ squamous cell carcinoma, cervix | 9 | 18 | 52 | – | 30 |

[a] $T_s$ = Duration of $S$ phase.

[b] LI = Thymidine labeling index (% of proliferative cells).

[c] $T_{cc}$ = Total proliferative cell cycle.

[d] Transit time = Viable differentiated cell compartment only.

Transit time studies have been used in several other benign dermatoses to estimate their degree of proliferative activity. Two of the four common types of ichthyosis, lamellar and epidermolytic hyperkeratoses, manifest a hyperplasia characterized by transit times of 4 days (6). The increased proliferation correlates clinically with extensive scaling or scale accumulation. The clinically milder forms of ichthyosis, vulgaris and sex-linked, have transit times similar to normal skin. Transit times have also been measured in human vagina and cervix as a function of ovarian function (Table 1) (4). In each condition the proliferative cell population alters its activity to maintain these constant transit times despite the different thickness of each epithelium.

The critical aspect of cell proliferation kinetics in any tissue is the measurement of the proliferative cell cycle. The first examination of cell cycle parameters in normal skin and psoriasis was reported by Weinstein & Frost (3). The thymidine labeling index of chronic steady-state psoriatic lesions is 22.7%, the $S$ phase is 8.5 hr, and the total proliferative cell cycle is 37.5 hr. In comparison, the normal epidermal cell cycle is 457 hr with a labeling index of 3.5% and an $S$ phase of 16 hr (Figure 1) (21, 22). No other benign dermatoses have as yet been investigated for complete cell cycle parameters in this manner. The much faster proliferative cell cycle in psoriasis produces the more rapid transit time of its differentiated cells and the clinical manifestation of increased scaling. The psoriatic cell cycle values should be noted for (*a*) the large proportion of proliferative cells in *S* phase, 22.7%, which makes this tissue particularly susceptible to chemotherapy with *S* phase-specific cell cycle drugs, and (*b*) the shortened *S* phase duration in comparison to cells of normal epidermis and basal cell carcinoma.

The cause(s) of increased proliferative activity in the benign dermatoses (psoriasis, exfoliative dermatitis, ichthyosiform dermatoses), whether examining the proliferative cell cycle or transit times, remains a complete mystery. There are no gross aberrations of cellular morphology as reproductive capability increases or any evidence of malignant degeneration. Increased cell proliferative activity can be provoked experimentally by diverse and even minimal types of skin injury, i.e. cellophane-tape stripping, ultraviolet light exposure, wounding, etc, in an effort to study the controlling factors of epidermal hyperplasia. A very active new field of research is the identification of a possible mitotic inhibitor control system commonly referred to as an epidermal chalone (23). A recent theoretical linkage between chalones and adenyl cyclase-cyclic AMP has been postulated by Vorrhees (24).

## CYTOKINETICS IN EPITHELIAL MALIGNANCIES

Although the cytokinetics of many types of experimental animal malignancies have been thoroughly investigated, there is a relative void of such knowledge in human tumors because of the inability to properly sample them. Because of their superficial involvement, cutaneous, and vaginal and cervical tissue malignancies have permitted experimental sampling to obtain significant information concerning human tumor cytokinetics. In 1968 Frindel et al determined parameters of cell kinetics in basal and squamous cell carcinomas following the systemic administration of 20 mCi of thymidine-$^3$H (25). Weinstein & Frost studied basal cell carcinoma cytokinetics using intralesional injections rather than intravenous tritiated thymidine (26). Results of these studies are presented in Table 1. A gratifyingly close correlation of *S* phase and labeling index values was obtained in the two studies confirming the validity of injection by either the systemic or intradermal technics.

The cytokinetics of malignant melanoma have had limited investigation. In 1969 preliminary studies in our laboratory projected an *S* phase of approximately 24 hr in metastatic melanoma lesions (27). Using intravenous tritiated thymidine Shirakawa et al found an *S* phase of 21 hr with a median cell cycle time of 72 hr (28).

Cytokinetics of squamous cell carcinoma in cervical and vaginal epithelium have been examined by Bennington (29) and Averette et al (30). Averette found that normal cervical and vaginal epithelial cells have an *S* phase of 9 hr and a cell cycle of 63 hr (4). However, squamous cell carcinoma of the cervix has a shorter cell cycle of 31 hr and a barely shortened *S* phase (30). Application of these data to chemotherapy is now being attempted (see below).

While the cell cycle parameters published on tumors probably reflect true proliferative activity, the values do not correlate well with the clinically observed doubling times of tumors. Whereas the basal cell carcinoma should double its cell population every nine days, the tumor itself is well known to take several months to double in size. It has therefore been proposed that cell loss through death must be an active ongoing process accounting for the clinically observed equilibrium (31, 32).

## CYTOKINETICS AND CHEMOTHERAPY

Current concepts of cancer chemotherapy are directed towards selectively attacking the rapidly proliferating malignant cell population without seriously affecting most of the normal (and slower) proliferating populations of the body (33). Such an effect requires knowledge of the cell cycle parameters in normal and malignant tissues. Concomitant information describing the action of chemotherapeutic drugs on the cell cycle is required. Bruce et al (34) have classified chemotherapeutic agents into 3 major classes: (*a*) cell cycle-nonspecific drugs such as nitrogen mustard that kill cells in all phases of the proliferative cycle; (*b*) cell cycle-specific drugs such as methotrexate and vinblastine that kill cells only in certain phases of the proliferative cycle, in this case $S$ and $M$ phase respectively; and (*c*) cell cycle-partially specific drugs such as 5-fluorouracil and cyclophosphamide that combine features of (*a*) and (*b*). Using this information an appropriate drug can be selected and dose schedules planned as a function of the cell cycle in the particular disease to achieve a much greater cell kill, if not cure (35, 36). Application to human malignancies is hampered primarily by a lack of accurate cytokinetic parameters in human normal and abnormal tissues (37).

An approach in this direction has been made to improve the treatment of severe psoriasis with methotrexate. Using the data on the rapid proliferative cell cycle of psoriasis (Figure 1), Weinstein & Frost proposed scheduling methotrexate administration over a period of 37 hr to completely expose an entire proliferative population of psoriatic cells while affecting only smaller portions of the other normal cell populations (21). In this manner a selective attack can be made on the abnormal tissue while sparing most of the organs from significant toxicity (38, 39). Based on the limited information of cytokinetics in two forms of ichthyosis, lamellar and epidermolytic hyperkeratoses, and pityriasis rubra pilaris, antimetabolite therapy has been used in a few patients with mixed results (40, 41).

Use of chemotherapy based on cytokinetics for solid tumors is still in its early investigative stages. Prospects in treating squamous cell carcinoma seem optimistic since this tumor is already known to respond to methotrexate when used in empirically devised schedules (42). In squamous cell carcinoma of the female genital organs, Averette et al have been testing multiple drug schedules containing methotrexate, hydroxyurea, and vinblastine based on cytokinetic data (30).

Cutaneous diseases have the unique advantage of permitting therapy by the topical application of medicaments. This would be of great value in treating psoriasis as the systemic toxic effects of methotrexate could be avoided if the drug were effective topically. Unfortunately none of the cancer chemotherapy drugs has as yet been successful when topically applied in psoriasis and this is one of the major frontiers of clinical and basic research in dermatology. Another disease suspected of having a cellular hyperplastic process, actinic keratoses, responds dramatically to the application of 5-fluorouracil (43, 44).

The study of benign and malignant cutaneous hyperplastic processes provides a valuable opportunity to investigate human cytokinetics and their response to therapy. A number of other diseases can be examined not only to obtain insights into their pathophysiology, but also to expand our knowledge of the role of chemotherapeutic drugs in any hyperplastic tissue.

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# THYROTROPIN RELEASING HORMONE: SECRETION AND ACTIONS[1]

7086

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## INTRODUCTION

Extensive physiologic and anatomic studies have demonstrated the central role of the anterior hypothalamus in the neurohumoral control of pituitary thyrotropin (TSH) secretion and biosynthesis (1, 2). The structural elucidation in 1969 of thyrotropin releasing hormone (TRH), isolated from more than 100,000 hypothalamic fragments derived from pigs (3) and sheep (4), constituted a momentous achievement in endocrine research. The subsequent large scale synthesis and availability of TRH has catalyzed vigorous activity in multiple areas of basic and clinical investigation. The progress made in understanding the structure, synthesis, secretion, degradation, and actions of TRH forms the substance of the present chapter. Excellent reviews on various aspects of TRH physiology have been published recently (5–9).

## TRH STRUCTURE-ACTIVITY RELATIONSHIPS

The simple structure of TRH and its strict configurational requirements for biologic expression make it an excellent molecule for studies of hormonal structure-function interrelationships. Native TRH is a weakly basic tripeptide with the structure L-pyroglutamyl-L-histidyl-L-proline-amide (Figure 1). Numerous analogues of TRH have now been synthesized. Almost any departure from the structure of native TRH has resulted in substantial if not complete loss of biological expression (5). The tripeptide L-Glu-L-His-L-Pro, devoid of normal C- and N-terminal modifications, is inert (10). Cyclization of the N-terminal glutamic acid to form the 2-pyrrolidone-5-carboxylic acid (PCA) group confers 0.1% of normal hormonal potency on this free-acid form of TRH (L-Pyro-Glu-His-Pro-O-H) (10). Modification of the C-terminal carboxyl group further enhances activity markedly, as exemplified by the analogue PCA-His-Pro-O-Me, which has about 49% of native TRH activity (10). Amino acid substitutions for the proline moiety yield compounds that are virtually inactive or inert (11, 12) and alterations in the PCA N terminus in the presence of the native proline-amide configuration similarly cause dramatic reductions in activity (13).

The central histidine residue is a critical determinant of TRH activity. The TRH diastereoisomeric analogue, in which the histidine residue is optically reversed to the D form, has only 3% of native potency (12), and photooxidation of the imidazole

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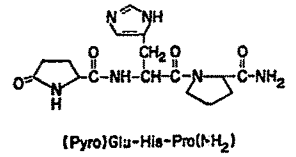

FIGURE 1. Structure of native TRH, L-pyroglutamyl-L-histidyl-L-proline-amide.

ring abolishes TRH activity completely (14). An important insight into TRH structure has been inferred recently from the discovery of two structurally related analogues involving modification of the histidine residue with markedly different biological properties (15). Methylation of the 1 position of the histidine imidazole ring yields a low potency TRH, whereas methylation of the 3 position results in a unique analogue with eightfold higher TSH releasing activity than native TRH (15). This increase in activity has been attributed tentatively to either the enhanced basicity of the imidazole 3 position or to a positive steric effect.

The biologic potency of all TRH analogues examined thus far has been correlated quantitatively with their respective binding affinities for pituitary TRH receptors (16, 17). No TRH analogues have been discovered to date which possess antagonistic activity to TRH.

## BIOSYNTHESIS OF TRH

The demonstration of TRH biosynthesis in vitro has furnished new insight into how this hypothalamic hormone is made and has provided a powerful new tool to examine TRH physiology (18, 19). Direct documentation of de novo TRH synthesis was obtained by incubating rat hypothalamic fragments with $^3$H- or $^{14}$C-labeled precursor amino acids. Ninety percent methanol extracts of tissues were then subjected to thin layer chromatography with multiple solvent systems, gel filtration with G-25 Sephadex, and CM-cellulose ion exchange chromatography. In all systems, identity of $R_F$ values was established for carrier synthetic TRH or $^{125}$I-TRH and newly formed isotopic TRH. Fragments derived from cerebral cortex, liver, kidney, and anterior pituitary, in contrast, failed to incorporate labeled precursors into TRH.

The mechanism by which TRH is synthesized is intriguing. TRH synthesis appears to occur enzymatically, analogous to the formation of the tripeptide glutathione and gramicidin S (19–21). Treatment with puromycin or RNase does not prevent TRH synthesis, and the TRH synthesizing system can be isolated in a ribosomal-free supernatant phase derived from hypothalamic homogenates centrifuged at 100,000×g. Optimum conditions for TRH formation require ATP and magnesium sulfate in addition to amino acid substrates. Glutamic acid, not pyroglutamic acid, is the N-terminus precursor, which suggests that the pyrrolidone ring is cyclized after formation of the dipeptide (Glu-His) or the tripeptide (Glu-His-Pro).

New concepts have emerged from the examination of various physiological determinants of the activity of hypothalamic "TRH synthetase" (19). Animals subjected to lower ambient temperature show increased TRH synthetase activity, in accord with known activation of the pituitary-thyroid axis following cold exposure (see section on TRH secretory physiology). Second, animals rendered hypothyroid have reduced TRH synthesizing activity, and animals rendered artifically hyperthyroid exhibit an accelerated rate of TRH formation.

From such data, Reichlin and collaborators have speculated that the set point controlling the rate of pituitary TSH secretion is the net result of a duality of thyroid hormone expression: positive feedback upon TRH synthetase and negative feedback upon TRH at the level of the pituitary thyrotroph (19). However, since stimulation of protein synthesis is fundamental to the biochemical expression of thyroid hormones in almost all cellular systems examined (22), the specific significance of thyroid activation of TRH synthetase in the control of the pituitary-thyroid axis needs to be established. However, the requirement of thyroid hormones for the induction of a normal hypothalamic-pituitary-thyroid hormone axis during amphibian metamorphosis has been inferred from the discovery that TRH synthetase is enhanced greatly during the transition from tadpole to frog (19). Finally, an examination of hypothalamic neurotransmitter influences upon TRH synthetase has revealed that both dopamine and norepinephrine can stimulate the release of labeled $^{14}$C-TRH from mouse hypothalamic tissue. This is of theoretical interest since those loci concerned with caloric homeostasis, which share anatomic proximity to the anterior hypothalamic region involved in thyroid regulation, are primarily dependent also on noradrenergic input (23).

Precise anatomic localization of TRH synthesizing activity is under study. TRH synthetase has been identified in extracts from the median eminence, and ventral and dorsal hypothalamus, but not in extracts from the preoptic area or the region of the mammillary bodies (24). These results generally agree well with known hypothalamic loci that elicit TSH secretion after electrical excitation and contain TRH biologic activity (25). However, since electrical stimulation of the preoptic area activates TSH secretion but does not have TRH synthetase activity, the preoptic area presumably furnishes neural input to peptidergic neurons involved in TRH formation and secretion into the hypophysial portal system.

## TRH DEGRADATION

The availability of isotopically labeled forms of TRH and the development of a specific radioimmunoassay for TRH have provided necessary tools for examining the metabolic fate of TRH, and preliminary studies have been reported. The clearance of exogenous $^{14}$C-TRH from plasma in the rat is exceedingly rapid, with an observed half-life of 4.16 min (26). The true half-life of plasma TRH is undoubtedly of even shorter duration, since elegant immunoassay studies of TRH degradation by plasma have revealed that approximately 90% TRH is inactivated after 60 min at 37°C (27). This inactivation process appears to be enzymatic in nature. It can be prevented by heating plasma at 60°C for 30 min and can be competitively inhibited to some extent by analogues of TRH exhibiting the common N-terminus structural sequence L-Pyro-Glu-His (28). Inactivation can be prevented completely by the introduction of the enzyme inhibitor 2,3-dimercaptopropanol (28). Rapid deamidation at the prolyl end of TRH appears to be the primary product of plasma catabolism of TRH, and the recovery of $^{14}$C derived from $^{14}$C-TRH in urine has yielded primarily two compounds, $^{14}$C-TRH and the $^{14}$C free-acid derivative (26, 29). Tissue-to-plasma ratios of isotopic TRH have indicated that TRH is concentrated in the anterior pituitary, liver, and kidney (30). In other studies where autoradiography has been used to identify organ distribution of radioactive TRH, concentration of TRH in the adenohypophysis was confirmed; but, in addition, TRH was discovered to be concentrated in other ectodermal structures including skin, salivary and lacrimal glands, and intestinal mucosa (31).

The kinetics of TRH distribution, degradation, and excretion in man have not been examined. It is clear, however, that rapid inactivation of TRH by human serum (27) and its probable very low concentrations in the extracellular compartment pose formidable obstacles toward ultimate quantitation of TRH in human plasma. However, under the special experimental circumstances of subjecting thyroidectomized, hypophysectomized rats to cold exposure, TRH biologic activity has been identified in the peripheral circulation (32).

## TRH SECRETORY PHYSIOLOGY

The control of TRH secretion until now has been inferred primarily from indirect measurements of serum TSH or circulating thyroid hormones in response to various physiological manipulations. Preliminary studies have been carried out, however, in which TRH biologic activity has been documented directly in the rat hypophysial portal system (33). Such portal plasma TRH activity can be augmented by unipolar electrical stimulation of the anterior hypothalamus and cannot be reduced by prior parenteral administration of L-triiodothyronine ($T_3$) (33). These results have provided direct support to the classical concept that the hypothalamic-pituitary-thyroid axis is a dynamic negative-feedback system involving thyroid hormone antagonism of TRH action upon the thyrotroph cell, not upon TRH secretion (1, 34).

A number of determinants influence pituitary TSH release through presumptive alterations in the rate of TRH secretion. First, the hypothalamic-pituitary-thyroid axis may participate in homeostatic adjustments attending states of increased calorigenic need. Cold exposure is capable of inducing a rapid elevation in serum TSH in animals, human neonates, and infants (19, 35, 36). Experimental studies in the rat have implicated both hypothalamic thermal receptors and peripheral cold receptors in the temperature-dependent activation of the hypothalamic-pituitary-thyroid axis (19). During chronic cold exposure, however, an increase in the rate of peripheral $T_4$ turnover may serve as an additional mechanism for augmented TSH secretion (37). Finally, the fall in serum TSH in subjects with primary hypothyroidism following the administration of bacterial pyrogen may also reflect thermal pyrogen influences upon TSH regulation (38).

A second stimulus to TRH secretion in man, independent of ambient temperature, is birth (39). Serum TSH rises rapidly to attain peak plasma concentrations at 2 hr following parturition. Such serum TSH elevations probably account for the increase in $^{131}$I-thyroidal uptake and serum-free thyroxin concentrations characterizing the early neonatal period, but their exact physiologic significance is not understood.

Three inhibitory stimuli to TSH secretion in addition to pyrogen have been identified. First, following acute oral glucocorticoid administration, serum TSH is rapidly lowered in man (40). Since this phenomenon can be simulated in rats whose pituitaries retain full responsivity to TRH in vivo or in vitro, it has been inferred that glucocorticoids must exert their acute inhibitory effects at a suprahypophysial level (40). In contrast, however, chronic glucocorticoid administration in man may result also in a blunting of pituitary responsiveness to exogenous TRH (41). Second, nonspecific stress can lower TSH secretion in rats, coincident with stimulation of ACTH release (42). This phenomenon could be a manifestation also of corticoid inhibition. Third, the catecholamine precursor L-dopa reduces serum TSH in hypothyroid subjects (43). Whether this is due to dopamine or norepinephrine inhibition of endogenous TRH release is presently conjectural. There is, as yet, no evidence supporting the existence of a "TSH-inhibiting factor," analogous to that for prolactin, whose secretion is activated by a dopaminergic mechanism.

Finally, in contrast to other hypothalamic control systems, TRH secretion does not appear to undergo circadian periodicity and is not affected by feeding, fasting, posture, or physical activity (38, 44).

## Mechanism of Action of TRH

Present knowledge of how TRH acts upon the anterior pituitary is highly incomplete, but the TSH secretory process appears to share features common to other endocrine and nonendocrine structures characterized by bulk storage of product in the form of secretory granules and whose contents are extruded by "emiocytosis" (5). TRH-mediated TSH secretion is dependent upon metabolic energy (45) and the availability of extracellular calcium ion (5) and TRH can stimulate glucose-6-$^{14}$C oxidation to $^{14}CO_2$ in isolated adenohypophyses derived from hypothyroid rats (46). Integrity of protein synthesis is not a requirement for the acute secretion of TSH, which agrees with the availability of abundant preformed hormone (45). In contrast, however, to secretion by the thyroid and salivary glands, TSH release has not been impaired by substances such as colchicine which interfere with the function of microtubules, even though tritiated colchicine can enter the anterior pituitary and binds to specific microtubular protein (47).

Elegant studies have revealed that the primary event in the action of larger peptide hormones such as ACTH, insulin, and glucagon is attachment to specific receptors on the cell surface of respective target organs (48–50). The recent availability of very high specific activity tritiated $^3$H-TRH (40 Ci/m*M*) that possesses normal biologic activity has permitted examination of potential TRH interactions with the anterior pituitary. Investigation by our group and others has documented selective and saturable binding of TRH in vitro in adenohypophyses derived from the rat, cow, and mouse pituitary TSH-secreting tumor of Furth (51–53). Moreover, under conditions of artificial hypothyroidism attended by an increase in the size and number of TSH-secreting cells, there occurs an enhancement in TRH binding, which suggests that TRH binds selectively to thyrotrophs (17, 53). Because the pituitary TRH receptor has been co-purified with the activity of two enzymes (5′-nucleotidase, adenyl cyclase) associated with the plasma membrane, TRH receptors, like those for larger protein hormones, are thought to be located on the cell surface (53, 54). Considerable interest has been focused on the general concept that TRH may activate adenylate cyclase and that cyclic AMP could then subserve as the intrapituitary messenger of TRH action. Although indirect pieces of evidence support this concept, no direct documentation of TRH stimulation of this pituitary enzyme has been achieved. It has been established, however, that pituitary secretion can be stimulated in vitro by agents which elevate the intracellular concentration of pituitary cyclic AMP, including theophylline, an inhibitor of pituitary cyclic nucleotide phosphodiesterase, prostaglandins of the $PGE_1$ series, and by introduction of the analogue dibutyryl cyclic AMP into the incubational medium (5, 55). Moreover, the prostaglandin analogue, 7-oxa-13 prostynoic acid, a known antagonist of prostaglandins in smooth muscle and the ovary, can inhibit TRH-mediated TSH release (56). Finally, crude hypothalamic extracts can stimulate pituitary adenyl cyclase and augment the intracellular concentration of cyclic AMP concomitant with an increase in pituitary secretion of GH and TSH (57). The failure to disclose similar effects with purified synthetic TRH could be a reflection of the small percentage of total pituitary cells involved in TSH secretion. Labrie and associates have demonstrated the presence of a cyclic AMP-dependent protein kinase in bovine pituitary tissue that is widely distributed among pituitary subcellular particulate and soluble fractions (58). Further studies are now needed to clarify how protein phosphorylations might participate in the process of TSH secretion and synthesis.

Antagonism of TRH action by thyroid hormones, in contrast to TRH-stimulated TSH secretion, requires pituitary protein synthesis, since thyroxine blockade can be prevented by pretreatment with actinomycin D (5). Although it has been speculated that a specific pituitary inhibitory protein to TRH is induced by thyroid hormones (5), such inhibition by thyroactive substances is not confined to TRH. TSH stimulation by the nonspecific pituitary secretogogue theophylline can also be blocked by $T_4$ in vitro (55). Pituitary inhibition by thyroid hormones may be mediated exclusively by $T_3$ derived from either extracellular $T_3$ or $T_3$ generated by $T_4$ monodeiodination, since a specific and saturable receptor for $T_3$, but not for $T_4$, has been demonstrated in the rat pituitary (59). Moreover, rat pituitaries bind $T_3$ 9.8 times more avidly than $T_4$ (59).

## TRH ACTIONS IN MAN

Since the initial report by Bowers et al that highly purified porcine TRH could stimulate TSH secretion in hypothyroid humans (60), the growing availability of synthetic TRH and radioimmunoassays for TSH has stimulated an enormous number of clinical investigations of TRH actions in man in health and disease.

*TSH release after TRH in normal subjects.*—TRH is a rapid and potent stimulus to TSH in man. In a large multiuniversity study, intravenous TRH was administered (100–800 μg) to 79 normal male subjects. A prompt increase in serum TSH to a mean peak concentration 322% above control was observed at 20–30 min (71) (Figure 2). Similar results reported by many groups have established the potential clinical utility of TRH in the evaluation of pituitary TSH-secretory reserve (9, 61–68). The intravenous dose-response function of TRH-mediated TSH secretion in normal men and women exhibits linearity between 62.5–500 μg

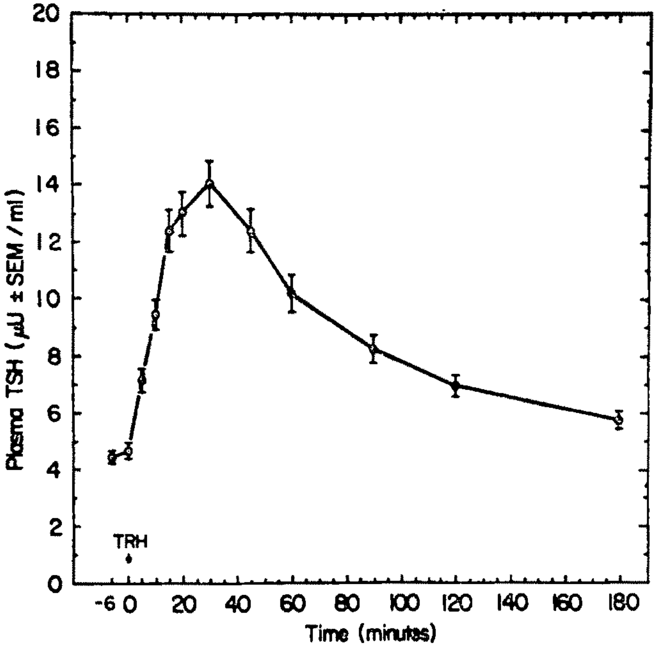

FIGURE 2. Mean plasma TSH responses to intravenous TRH in 79 normal subjects. Vertical bars represent one standard error of the mean (From Anderson et al 1971. *N. Engl. J. Med.* 285:1279–83).

TRH (63). Although maximal TSH responsiveness has been found to be slightly greater in females, this difference was statistically different ($p < .05$) at only one dose (62.5 µg). Interestingly, when TRH responses have been compared in man and woman as a function of age, TSH maxima decline in men but not women in the age ranges 40–59, and further at 60–70 (69). The role of sex steroids in this differential response has not yet been established. Repetitive administration of the identical quantity of TRH to single subjects has revealed that variation in TSH patterns can be appreciable, with a coefficient of variation as great as 40% (67). Repeated administration of 62.5 µg or 500 µg of TRH on five consecutive days in normals resulted in obtundation of TSH secretion by the third or fourth day of testing (63). This progressive diminution in TSH secretion after TRH may be a reflection of pituitary blockage by the concomitant increase in serum thyroid hormones, particularly $T_3$ (70), but the possibility of depletion of a finite "releasable pool" of pituitary TSH has not been excluded.

*Specificity of TRH activity in normal man.*—The initial presumption that TRH stimulated TSH secretion specifically in man was based on the failure of TRH to release follicle stimulating hormone, luteinizing hormone, growth hormone (HGH), or cortisol (71). However, when immunoassay methodology for quantitation of prolactin (HPr) in human sera became available, it was apparent that TRH is a very potent HPr secretogogue (72–74). (A more detailed discussion of

TRH in relation to HPr secretion is treated elsewhere in this volume by H. G. Friesen.) In addition, in some normal subjects plasma HGH may become elevated to variable degrees between 120–180 min following TRH (75). This HGH response, however, is not coincident with that of TSH and HPr, and may be nonspecific. In striking contrast to normals, however, HGH secretion can be stimulated by TRH in a high percentage of acromegalic subjects (76).

*Secondary TRH influences upon thyroid physiology.*—Following intravenous TRH, acute rises in serum $T_3$ have been reported by Hollander et al (70). Smaller elevations in serum $T_4$ have been reported also in a small number of subjects, but statistical treatment of these data were not included (77), and confirmation of this phenomenon was not obtained in the larger multiuniversity study previously mentioned (71). In contrast, a sufficient dosage of oral TRH can evoke unequivocal elevations in both serum thyroxine and thyroidal uptake of radioiodine (78–81). Following a single oral dose of 20 mg TRH, serum $T_4$, measured by displacement analysis, consistently rose to a maximum at 6–9 hr (81). This single oral schedule of TRH did not alter thyroidal handling of radioiodine. However, when 80 mg TRH was administered every 12 hr on five successive occasions, an increase in radioactive uptake was found in ten subjects, ranging from 60–500% above baseline (78). These observations agree with the well-known phenomenon of the greater sensitivity of thyroidal release than thyroidal uptake to TSH stimulation. Qualitatively similar alterations in serum $T_4$ and $^{131}I$ uptake by the thyroid have been reported by another schedule of orally administered TRH (20 mg each 8 hr × 48 hr) (79). A dose-response function for oral TRH and serum TSH has been described with lower dosages of TRH (1–8 mg at 3–5 day intervals) that produced progressive increments in TSH, serum $T_4$, and radioactive inorganic iodide, but increments in thyroidal radioiodine uptake and serum protein-bound $^{125}I$ levels were not detected using this smaller dosage regimen (80).

These reports of oral TRH stimulating measurable thyroidal events lend encouragement toward the development of tests of pituitary-thyroid reserve with potentially much wider accessibility than those dependent upon TSH radioimmunoassays.

*Untoward effects of TRH.*—Careful assessment of hematologic, renal, and hepatic status on TRH recipients has uncovered no objective toxic effects from acute intravenous administration of TRH except occasional slight diastolic hypertension (71). However, mild transient subjective symptoms have been noted in about 70% of subjects, consisting most commonly of nausea, a flushed or queasy sensation, and an urge to urinate. The frequency of these symptoms appears to be dose-related. Virtually no symptoms occur, by contrast, after intramuscular or oral TRH administration (75, 80).

*TRH in hypothyroid states.*—In patients with documented primary hypothyroidism TRH administration uniformly produces a greater than normal TSH secretory response (9, 64, 65, 68, 75, 82). Thus, elevated serum TSH concentrations characteristic of primary myxedema do not reflect maximal pituitary secretory capacity. This augmentation of TRH action may simply be a manifestation of the increased number of pituitary TSH secreting cells, whose unit response remains normal. When hypothyroidism, in contrast, occurs in the setting of a pituitary tumor or ischemic necrosis (2° hypothyroidism) most investigators have found invariably reduced or absent TSH release after TRH administration (63, 71, 75, 82, 83). One group, however, has reported "prolonged or exaggerated" responses after TRH in patients with pituitary tumors (84). The pathophysiologic significance of this anomaly is not presently known. However, since pituitary TSH secretory reserve is often impaired in patients with pituitary tumors prior to the development of hypothyroidism (62), this accentuation of TSH secretion after TRH in nonprimary hypothyroidism is more consistent with an impairment in endogenous TRH availability than pituitary thyrotroph failure. In this regard, the concept of TRH deficiency (hypothalamic hypothyroidism or tertiary 3° hypothyroidism) was proposed to explain the intact TSH secretory pattern after TRH in a patient with idiopathic hypopituitarism (85). This notion has been extended now to children with idiopathic hypopituitarism, most of whom interestingly exhibit preservation of normal TSH response to TRH (86, 87). If a "hypophysiotropic" basis can be documented for other tropic hormone deficiencies in this important patient population, therapy for growth failure and sexual immaturity with releasing hormones is promising. In subjects with "isolated TSH deficiency," two patterns of response to TRH have been described. Two sisters of a consanguineous marriage with hypothyroidism failed to exhibit any rise in TSH after TRH, which suggests a pituitary lesion in the formation or secretion of TSH (88). In contrast, a disturbance in the production or transmission of TRH has been postulated in a 19-year male with a normal TSH response to TRH (89). Undoubtedly, "isolated TSH deficiency states," like Bright's disease, will emerge ultimately as a complex spectrum of pathophysiologic mechanisms as further patients are analyzed.

A potential experimental model for studying TRH deficiency has been described recently in a genetic form of obesity in the rat (90).

*TRH in hyperthyroid states.*—It has been clearly established by many groups that states of overt thyrotoxicosis with elevations in total free $T_4$ and/or total free $T_3$ cause blunting or complete blockage of TRH-mediated TSH release (9, 64–66, 68, 75). More enigmatic, however, is the observation that subjects with "euthyroid" Graves' disease may also fail to respond to exogenous TRH in the face of apparently normal concentrations of $T_4$ (91). Evidence is emerging, however, that very small increments in circulating thyroid hormone can induce alterations in TRH expression. In subjects on chronic oral replacement therapy, Snyder & Utiger have shown that the daily administration of only 15 $\mu$g $T_3$ and 60 $\mu$g $T_4$ is accompanied by a 75% reduction in TSH response to 400 $\mu$g TRH IV, a replacement schedule that caused only a small rise in serum $T_3$ and no detectable increase in serum $T_4$ (92). Moreover, our group has reported that a single dose of a synthetic mixture of $T_4$ and $T_3$ can effect complete TRH inhibition after 48 hr when serum $T_4$ is normal and serum $T_3$ has returned to near normal concentrations (93). These data may provide an explanation for the failure of subjects with euthyroid Graves' disease to respond to TRH. Their circulating thyroid hormone concentrations, albeit in the normal population range, may be elevated sufficiently to restrain thyrotroph function. Moreover, the dysynchrony between peak serum $T_3$ and peak inhibition of TRH action suggests that augmented pituitary thyroid hormone turnover, and not absolute serum thyroid hormone concentrations, controls TRH expression. Inhibition of TRH effect by thyroid hormones has been shown not to relate specifically to their calorigenic action, since comparable enhancement of calorigenesis by salicylates does not reduce TSH secretion in man (94).

*TRH in euthyroid subjects with pituitary tumors.*—The potential value of TRH in uncovering subtle reductions in TSH reserve in the context of euthyroidism has been demonstrated nicely in the large study of Fleischer et al (83). Thirty subjects with pituitary tumors and enlarged sella turcicas have been examined, of whom only four were hypothyroid by clinical and laboratory criteria. A spectrum of responses to 0.5 mg TRH was uncovered, including absent TSH secretion in six patients, impaired release in eight, and intact responsiveness in only twelve. Thyroid function was similar among TRH responders and nonresponders. Similar results have been reported by Hall and associates (95).

*Pharmacological modifications of TRH stimulation in man.*—Preliminary observations have been recorded which indicate that pituitary responsiveness to TRH can be influenced by certain nonthyroidal determinants. First, theophylline augments TRH stimulation of TSH release (96). The possible relationship of this phenomenon to increased pituitary cyclic AMP in man is not known. Second, TRH-mediated TSH release has been allegedly enhanced in both males and females given 8 mg estradiol for five consecutive days (97). Caution should be exercised, however, in ascribing this to a specific estrogen effect since the greatest percentage TSH change was within the range of variation that can be encountered in repetitive studies in a single subject (67). Third, L-dopa administration, in addition to its reduction of endogenous TSH in hypothyroidism, has been shown to cause inhibition of exogenous TRH action (98). Whether this effect is due to L-dopa itself or to derived metabolites, such as dopamine and norepinephrine, is presently under study.

*Summary of TRH clinical applications.*—Extensive studies of TRH actions in man have documented the diagnostic usefulness of this exciting new tool in the evaluation of the hypothalamic-pituitary-thyroid axis. TRH would appear to be particularly helpful in the recognition of subtle disturbances in TSH and HPr secretory reserve prior to the development of overt pituitary deficiency states, and in the differentiation between hypothalamic and pituitary causes for hypothyroidism. Finally, the exquisite sensitivity of the thyrotroph to inhibition by thyroactive substances may make the loss of normal pituitary responsivity to exogenous TRH an important new criterion for the identification of thyrotoxic states in patients with questionable thyroid hormone excess.

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# RENAL ENERGY METABOLISM AND SODIUM REABSORPTION

7087

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## INTRODUCTION

The kidney is particularly intriguing to those concerned with organ metabolism. It has all the superficial features needed to conduct quantitative function studies; a single artery and vein permit measurement of extractions from and additions to blood perfusing the organ. The single artery also provides a route whereby the chemical environment of the kidney can be altered by administration of substrates or compounds that alter substrate metabolism. It has a single product, the urine, whose volume and composition can be measured in calculation of work. Lastly, as a paired organ, it offers the experimental tactic in which one kidney may serve as an ongoing control.

In the above qualities, the kidney approaches the ideal experimental preparation. But there is an inevitable complexity; in a metabolic sense the kidney consists of at least two distinct tissues, cortex and medulla. Function studies in the intact organ measure the net of both cortical and medullary metabolism. The ideal is diminished further because for some substances (e.g. glucose), renal uptake and production occur concomitantly. Despite these complications, metabolic studies in vivo have enriched our knowledge of renal function.

Renal function is initiated by delivery to the kidneys of a flow of blood amounting to 20–25% of the cardiac output. Of this, 20–35% of the plasma is filtered through glomerular capillaries. The filtration process is passive; its driving force is generated by the heart. Because of the permeability properties of the glomerular membrane, proteins and other high molecular weight components are virtually excluded from the filtrate. However, large quantities of protein-free plasma enter the tubules and are available for excretion. Delivery of such large quantities of fluid requires that the kidney continuously reclaim 98–99% of the filtrate; herein lies the need for direct metabolic energy. Although reclamation of filtered substances such as bicarbonate, glucose, potassium, phosphate, and amino acids is crucial to the economy of the body, reabsorption of sodium, chloride, and water represents the most important regulatory function in a quantitative sense.

Reabsorption of sodium by the kidney is an energy-dependent process. Data obtained by the technique of micropuncture (1) support the thesis that filtered sodium passes freely through the luminal membrane of the proximal tubule down an electrochemical gradient. Return of sodium to the circulation is accomplished by transport across the antiluminal (basal) membrane against an electrochemical gradient. The energy required for this active transport of sodium is supplied by metabolic processes occurring within the tubule cells. On a stoichiometric basis, sodium reabsorption accounts for about 70% of all renal transport activity.

We shall review the relationships between energy metabolism of the whole kidney and the reabsorption of filtered sodium. Since this topic has not been previously discussed in the *Annual Reviews*, sources from the literature of the past 12–15 years have been selected. Efforts have been made to represent the variety of views held by workers in this field. The reader should appreciate that much significant work concerning the relationship of metabolism to transport activities of kidney tissue slices in vitro has not been cited. These contributions have been extremely valuable but it is uncertain whether renal transport processes in vitro can be compared to those occurring in vivo (2). The intact mammalian kidney reabsorbs nearly 80 meq Na/g kidney/day across the renal tubule cells. Such *transcellular* sodium transport does not occur in kidney tissue in vitro, while *transmembrane* sodium transport by kidney tissue may be no different from that occurring in other cellular tissues. Hence, there is reason to believe that the relationship between ion transport and energy metabolism in vitro cannot be directly extrapolated to that of the intact organ. In the present review, metabolic data obtained in vitro are intended to complement information derived from studies of intact functioning kidney.

In limiting this discussion to the special relationship between sodium reabsorption and renal energy metabolism, we are also necessarily omitting important information on renal metabolism relating to other functions of the intact kidney such as tubular transport of organic acids, hydrogen ion secretion, and the production of ammonia. A comprehensive review of this latter topic has appeared recently (3). In addition, the reader is directed to reviews of general renal energy metabolism by Metcoff & Yoshida (4), human kidney metabolism by Wesson (5), renal biochemical pharmacology by Landon & Forte (6), and the relationship between fasting and mineral metabolism (7).

## RENAL SODIUM REABSORPTION AND OXYGEN CONSUMPTION

The concept that renal sodium reabsorption ($R_{Na}$) is an energy-dependent process gains considerable support from studies revealing a direct relationship between $R_{Na}$ and renal oxygen consumption ($Q_{O_2}{}^{Ren}$) in dog (8–12) and rabbit (13). Such experiments, using one kidney, require measurement of serum and urinary sodium, glomerular filtration rate (GFR), renal blood flow, and both arterial (A) and renal venous (RV) oxygen content. Renal oxygen uptake is derived from the renal blood flow and A−RV difference in oxygen content. $R_{Na}$ is estimated as filtered sodium minus excreted sodium. The molar ratio of $R_{Na}/Q_{O_2}^{Ren}$ obtained by different investigators is in the range of 18–35.

*Effect of physiologic maneuvers.*—In a classic study Lassen, Munck & Thaysen (8) demonstrated the constancy of $R_{Na}/Q_{O_2}^{Ren}=28$ as $R_{Na}$ was diminished in association with decreases in GFR induced either by bleeding or renal vein obstruction. When glomerular filtration was abolished during exsanguination, the "basal" oxygen consumption was 1.0 μmole/g tissue/min, 15% of $Q_{O_2}^{Ren}$ in the control state (8). A similar linear relationship has been reported between spontaneous values of $R_{Na}$ observed in dogs and simultaneously measured rates of renal oxygen consumption (9). The low value of observed (8) or extrapolated (9) renal oxygen consumption when glomerular filtration is zero suggests that the greater part of renal oxygen consumption results from the energy needs for sodium reabsorption. The relationship between $R_{Na}$ and $Q_{O_2}^{Ren}$ remains constant as $R_{Na}$ is increased in dogs by increasing GFR following the administration of glycine (10), or when decreased $R_{Na}$ is induced by increasing ureteral pressure (11).

In more recent studies, renal energy metabolism has been monitored in vivo by measuring the rise in local intrarenal temperature with a thermistor probe just after renal arterial occlusion abolishes renal blood flow. In the absence of circulation, heat generated by ongoing metabolic processes is not dissipated and the rate of local temperature rise is proportional to the local metabolic rate (14). Such studies have been useful in distinguishing the behavior of renal medulla from that of cortex in vivo since probes can be selectively placed. Constriction of the renal artery caused a parallel reduction in $R_{Na}$ and the rate of heat accumulation in both cortex and medulla (15). This suggests that in medulla as well as cortex, sodium transport is closely linked to energy metabolism.

Sodium reabsorption and oxygen consumption can, however, be dissociated. During natriuresis evoked by mannitol infusion in dogs, net reabsorption of sodium declines as much as 34% (11) from control values, while $Q_{O_2}^{Ren}$ remains unchanged (10, 11, 16). The ratio of $R_{Na}/Q_{O_2}^{Ren}$ falls accordingly from 29 to 20 (11, 16). It has been suggested (11) that this decrease results from enhanced delivery of sodium to the ascending limb of the loop of Henle, where transport work might be more costly in terms of energy requirements. However, the oxygen cost of sodium transport appears to be similar for proximal and distal parts of the nephron (16). An alternative explanation is that this effect results from sodium backflux requiring active reabsorption (10) not accounted for in the calculation of $R_{Na}$ = filtered Na − excreted Na. Comparable experiments by Kiil et al (15) in which renal heat accumulation was measured during mannitol diuresis showed that cortical metabolism was unaffected while that of outer medulla increased. This was explained on the basis of increased medullary sodium reabsorption related to increased delivery of filtered sodium to the ascending limb of the loop of Henle (15). The failure to detect increased oxygen consumption by the whole kidney during mannitol diuresis (11) may be explained by the fact that medulla accounts for only 30% of the renal mass. In addition, the oxygen tension and availability of oxygen is less in medulla than in cortex (17–19). Anaerobic glycolysis is accordingly more prominent in medulla (20–22) as will be detailed below.

*Effect of metabolic inhibitors.*—From the foregoing discussion it is clear that several experimental maneuvers will primarily reduce $R_{Na}$ and proportionately reduce renal oxygen consumption. Although mannitol diuresis represents an exception, the apparent inconsistency of its action with other maneuvers can be explained as mentioned above. Further evidence in support of a cause-and-effect linkage between $R_{Na}$ and $Q_{O_2}^{Ren}$ comes from experiments in which inhibition of energy metabolism results in proportional decreases in $R_{Na}$. The hypothesis underlying such experiments is that if oxidative metabolism is essential for renal sodium reabsorption, then procedures which decrease the rate of oxidative metabolism, or substances which block either the electron transport system (ETS) or the synthesis of adenosine triphosphate (ATP) should be natriuretic. Hypothermia, a maneuver expected to decrease the metabolic rate, decreases renal respiration and $R_{Na}$ (23). Recent studies in the rat have shown that the decreased metabolic rate associated with hypothyroidism is associated with an equivalent decline of tubular sodium reabsorption during saline loading (24).

Cyanide, a well-known inhibitor of cellular respiration, acts by blocking the transfer of electrons to $O_2$ at the site of cytochrome $a_3$ of the mitochondrial respiratory chain. A substantial ipsilateral natriuresis occurs when millimolar quantities of cyanide are infused into one renal artery of dogs (9, 25–28). The unilateral effect demonstrates that the natriuresis does not result from respiratory inhibition in other parts of the body. The resultant decrease in $R_{Na}$ is proportional to the decrease in renal oxygen uptake (9); the $R_{Na}/Q_{O_2}^{Ren}$ ratio is unchanged from control observations. The assumption that respiratory inhibition actually causes the decreased $R_{Na}$ has been further strengthened by the observation that hypoxia augments a cyanide-induced natriuresis without altering the $R_{Na}/Q_{O_2}^{Ren}$ ratio (27). Decreased sodium reabsorption has also been demonstrated by micropuncture techniques in kidneys of the rat (29–31) and *Necturus* (32) during cyanide administration.

Antimycin, inhibitor of the transfer of electrons from cytochrome *b* to cytochrome *c* of the ETS, has a potent ipsilateral natriuretic effect (26). Fluoroacetate, an inhibitor of the enzyme aconitase in the tricarboxylic acid (TCA) cycle, is also natriuretic (33). The effect of these agents upon renal oxygen consumption in vivo has not been studied.

Amobarbital inhibits electron transport from flavoprotein to coenzyme Q. Some investigators have reported a natriuresis after amobarbital administration (29) while others have observed no such effect (26). Malonate, a Krebs cycle inhibitor, and azide, which interferes with the ETS in high doses, do not alter sodium reabsorption or excretion (26). However, negative results with inhibitor compounds must be interpreted with caution; administration does not assure delivery of the inhibitor to an appropriate metabolic site. Apparent interference with coenzyme Q of the ETS by infusion of Qo, its benzoquinone nucleus, has resulted in sodium excretion of 9 to 17% of the filtered load on the infused side (9, 34). Because of a rise in GFR, $R_{Na}$ was unchanged (9). $Q_{O_2}^{Ren}$ was erratic but generally also unchanged (9). The antibiotic oligomycin that inhibits the phosphorylation of ADP and electron flow along the ETS (35) is natriuretic (33).

In summary, some compounds that inhibit the ETS in vitro reduce $R_{Na}$ concomitant with $Q_{O_2}^{Ren}$. Although the evidence is indirect, it is consistent with the hypothesis that renal sodium transport depends in part on oxidative metabolism as a source of energy.

## Renal Sodium Reabsorption and Substrate Utilization

Oxygen consumption by the kidney is necessarily a reflection of the oxidation of substrates for the production of energy. To be consistent with the hypothesis offered above, substrate utilization should bear some relationship to sodium reabsorption. As with oxygen consumption studies, A−RV concentration difference of a specific substrate is measured along with renal blood flow thus enabling the determination of the rate of net appearance or disappearance of substrate. Corrections for urinary water (36) and substrate loss may be required.

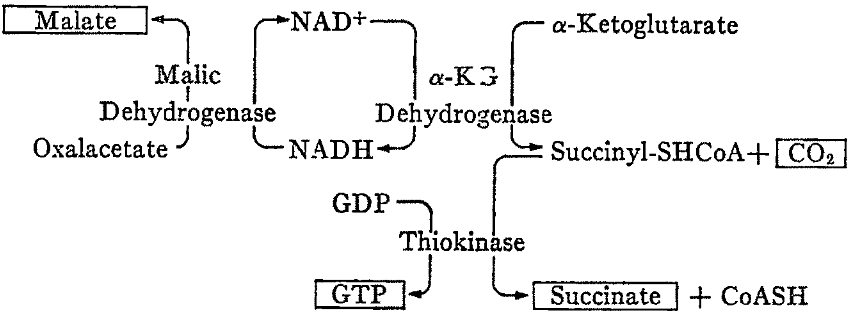
*Free fatty acids.*—Uptake of free fatty acid (FFA) by the mammalian kidney is evident in extraction ratio $[(FFA)_{A-RV}/(FFA)_A]$ of 5% (37–39), 12% (40), and 27% (41) obtained in dog (37, 40, 41) and human (38, 39). The A−RV difference for FFA is not related to the rate of renal blood flow (37). The uptake of FFA by kidney in vivo varies directly with the arterial concentration of this substrate during both acute saline (37, 41, 42) and mannitol diuresis (41, 42) in dogs, and under control conditions in humans (38).

The extraction of various free fatty acids by dog kidney has been studied by Gold & Spitzer (40). Of the FFA normally present in the plasma, significant renal extraction (15%) was found only for palmitate. Oxidation of infused $^{14}C$-palmitate was demonstrated by recovery of $^{14}CO_2$ from the renal vein. A net recovery of $^{14}CO_2$ did not occur with $^{14}C$-oleate, but did occur with $^{14}C$-octanoate. Palmitate therefore appears to be the only free fatty acid normally present in significant amounts in the plasma that can be utilized by the kidney of the dog. Palmitate is taken up by human kidney as well (43). Since oxidation of oleate does occur in slices of rat kidney cortex in vitro (44), extrapolation of such results from one species to another must be undertaken with caution.

Other substrates taken up by the kidney include lactate and pyruvate (38, 39, 41, 45, 46) and citrate (38, 41). Nine (39, 45) to 20% (41, 46) of ambient renal arterial lactate may be extracted by the kidney in dogs (41, 45, 46) and humans (39). Complete oxidation of lactate could account for over 20% of $Q_{O_2}^{Ren}$ (38, 45). Over 50% of $CO_2$ produced by the kidney may be derived from ambient lactate (46). Net uptake of pyruvate and citrate are considerably smaller. In studies on 71 human subjects, Nieth & Schollmeyer (38) observed a net uptake of citrate and pyruvate as well as FFA and lactate. Renal oxygen consumption was measured in these same studies and, assuming complete oxidation of these substrates, the calculated oxygen consumption accounted for 107% of that actually observed. Of this, 59% was contributed by FFA, 35% by lactate, 10% by citrate, and 3.5% by pyruvate. The assumption that substrate uptake by the kidney can be equated with substrate oxidation is, however, unwarranted as indicated by studies in vivo (39, 43, 46) and in vitro (44). No net extraction of glucose was apparent (38), a finding to be discussed in more detail below.

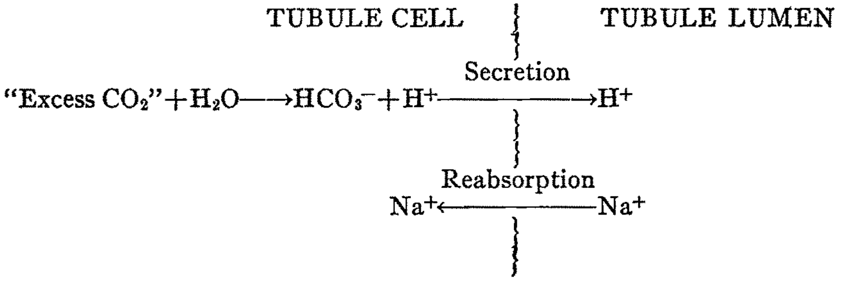
The ability of kidney cortex to utilize the ketone bodies β-hydroxybutyrate and acetoacetate has been documented for rat kidney in vitro (44), in the perfused isolated rat kidney (47), and by the intact dog kidney during β-hydroxybutyrate infusion (48). In the latter study, significant renal extractions of β-hydroxybutyrate and acetoacetate are related to their respective plasma concentrations, while the average extraction of each was 9% (48), findings similar to those for FFA in the dog (37, 41, 42). The affinity of the kidney was greater for β-hydroxybutyrate (48). Complete oxidation of the β-hydroxybutyrate could account for an average of 41% and acetoacetate for 11% of net renal oxygen consumption during such infusions (48). β-Hydroxybutyrate uptake by kidney of starved obese human subjects has been shown by Owen et al (39); one sixth of the uptake could be accounted for by production of acetoacetate.

If renal energy metabolism is utilized primarily for sodium reabsorption, as suggested by the oxygen consumption data already discussed, then a relationship might be expected between $R_{Na}$ and oxidation of specific substrates. This has not been directly measured. However, $R_{Na}$ has been related to renal substrate *uptake* in dogs (42, 49). Barac-Nieto & Cohen (42) demonstrated a direct relationship between uptake of ambient FFA and the spontaneous rate of sodium reabsorption. The relationship was such that 750 μeq of sodium were reabsorbed for each microequivalent of FFA taken up. If complete oxidation is assumed, this translates to a $R_{Na}/Q_{O_2}^{Ren}=30$, consistent with previous observations. However, previous reports by Cohen (50) and Barac-Nieto & Cohen (43) indicate that complete oxidation of FFA is not likely. Although other investigators (51, 52) have obtained values of 0.7 to 0.8 for the respiratory quotient (RQ) of the kidney, Cohen (50) observed mean values of 1.33 ± 0.07 in uninephrectomized dogs undergoing saline diuresis. Since the RQ for lipids is 0.7 and that for glucose and glucogenic substances is 1.0, a value above 1.0 implies nonoxidative $CO_2$ production. The following scheme for anaerobic $CO_2$ production was proposed (50):



The ability of kidney to carry out such reactions was previously shown by Hunter (53) and anticipated to occur in medulla with its low oxygen tension. However, the small contribution of medulla to the total metabolism of the kidney almost assured participation of the cortex as well (50). The sequence shown produces guanosine triphosphate (GTP) and utilizes ketoglutarate ($\alpha$-KG), a substrate for which the kidney has a specific affinity (54). This hypothesis has been strengthened by the work of Randall & Cohen (55) who observed significant $CO_2$ production from both cortical and medullary slices when high concentrations of substrates (OAA 0.42 m*M*, $\alpha$-KG 8 m*M*) were used. However, this rate of $CO_2$ production was less than the rate of excess $CO_2$ produced in vivo (50). The operation of other similar pathways was thought likely (55). Alternatively, the rate of metabolism in kidney slices is lower than that of perfused (47) or intact kidney.

An additional hypothesis arising from this work (55) was that excess $CO_2$ produced might participate in bicarbonate regeneration by the tubule cell. The resultant hydrogen ion might then be excreted as titratable acid in exchange for an equivalent amount of sodium.


TUBULE CELL
TUBULE LUMEN
Secretion
"Excess $CO_2$"$+H_2O \longrightarrow HCO_3^- + H^+ \longrightarrow H^+$
Reabsorption
$Na^+ \longleftarrow Na^+$

This exchange might be energized by the high energy phosphate (GTP) produced during the anaerobic decarboxylation. A moiety of sodium might thereby be reabsorbed without oxygen consumption.

*Lactate.*—Levy (45) demonstrated that A−RV lactate differences in normal dogs were not influenced by decreases in renal blood flow caused by renal arterial constriction. Renal lactate utilization was therefore proportional to renal blood flow (RBF). Changes in GFR and, therefore, in $R_{Na}$ are also proportional to changes in RBF. This suggests that net renal lactate uptake is proportional to $R_{Na}$ and, in fact, Dies et al (49) have demonstrated such a relationship. A linear correlation between $R_{Na}$ and $Q_{lactate}^{Ren}$ in saline-loaded dogs was observed as $R_{Na}$ was progressively decreased in response to increasing ureteral pressure; 1 $\mu$mole of lactate corresponded to reabsorption of 115 $\mu$mole of sodium. Again assuming complete lactate oxidation, the ratio $R_{Na}/Q_{O_2}^{Ren}$ equal to 38 was obtained, a value higher than those reported elsewhere for this ratio. The diversion of some lactate to nonoxidative pathways, i.e. glucose production (39, 45), would further increase the value of $R_{Na}/Q_{O_2}^{Ren}$. It is therefore likely that $R_{Na}$ is energized, at least in part, by oxidation of substrates other than lactate (39, 43).

In contrast to Barac-Nieto & Cohen (43), Dies et al (49) failed to demonstrate a significant correlation between $Q_{FFA}^{Ren}$ and $R_{Na}$. The authors suggested that lactate might be more available than FFA to an energy pool driving sodium transport. This suggestion has some basis in earlier work (47) in which utilization of acetoacetate by whole perfused rat kidney was the same per unit weight as that for rat kidney cortex slices, while the oxygen consumption in the perfused organ was twice as high. Since only the perfused organ engages in tubular sodium reabsorption, the oxidation of acetoacetate may relate only to nonsecretory, i.e. reabsorptive, function (47). Assuming that acetoacetate and FFA share the same metabolic pathways, oxidation of FFA may be similarly unrelated to sodium reabsorption. However, this hypothesis is not supported by the findings of Owen et al (39) in long-fasted (5 weeks) obese human subjects. In these patients data obtained by arterial renal vein catheterization showed that all of the measured glucogenic substances (lactate, glycerol, and amino acids) taken up by the kidney could be accounted for by renal glucose production.

A completely different approach to the relationship between sodium reabsorption and substrate utilization has been taken by Waugh & Kubo (56, 57) who suggested that substrate limitation might be responsible for the decreased fractional sodium reabsorption observed during saline loading (58). They observed that such expansion natriuresis in five dogs was markedly blunted by the inclusion of lactate 2 m*M*, pyruvate 2 m*M*, and $\alpha$-ketoglutarate 4 m*M* in the saline infusion of dogs pretreated with desoxycorticosterone acetate (DOCA) and vasopressin (56). However, in further studies (57), the plasma concentrations of lactate, FFA, and citrate were measured before and after saline expansion. No change was observed, though the expected natriuresis occurred. If it can be assumed that renal blood flow did not fall, then there was no decline in delivery of substrates to the kidney. Renal substrate uptake was not measured. Furthermore, from data presented, it is apparent that $R_{Na}$ increased an average of 11% after expansion. The results are uninterpretable with respect to any relationship between substrate utilization and $R_{Na}$.

In summary, clear relationships between the utilization of specific substrates and $R_{Na}$ have not emerged. In view of the findings here discussed that renal substrate utilization appears to be partially dependent upon plasma substrate availability and that a variety of substrates may be utilized, it is apparent that the kidney is flexible with respect to fuels metabolized in support of the constant energy requirements for sodium reabsorption

*The "glucose problem."*—Net renal uptake of significant quantities of glucose in vivo has not been observed at ambient levels of blood glucose (38, 39, 41, 59–61). In studies of Bollman & Grindlay (59), as well as those of Dies et al (41), some dogs showed small net renal glucose uptake, while in others there was a small net release. In similar studies of McCann & Jude (60), small but consistently negative A−RV glucose differences were observed in fasted, unanesthetized dogs with previously placed indwelling vascular catheters. In eight animals the average A−RV glucose difference of −1.9±0.46 mg/100 ml suggested net renal gluconeogenesis. It was estimated that 9–18 mg glucose/kg body weight/hr could be produced by the kidney.

Renal gluconeogenesis from endogenous substrates has been observed in rat cortex slices and increases markedly after pretreatment of the rats with a low carbohydrate diet (62). The data of Owen et al (39) in fasted obese human subjects reveal a net renal glucose production of nearly 40 g/day—a value equaling 45% of total body gluconeogenesis. Because of net renal gluconeogenesis and the fact that uptake of lactate, glycerol, and $\alpha$-amino acids can be accounted for by glucose release (39), FFA and/or ketone body utilization must give rise to energy for gluconeogenesis *and* presumably Na reabsorption as well. This constitutes evidence against the hypothesis cited earlier that FFAs do not contribute to $R_{Na}$ (47). Nor is it consistent with the finding that $Q_{FFA}{}^{Ren}$ does not correlate with $R_{Na}$ in the fasted dog (49).

Renal gluconeogenesis in vivo from ambient substrates has been documented widely in relation to ammonia production by the kidney (3). When arterial lactate concentrations are increased by its infusion, the net uptake of lactate by the kidney increases proportionately (41, 45). This increased uptake can be quantitatively accounted for by net renal glucose production (41). Renal gluconeogenesis from tracer quantities of $^{14}$C-labeled $\alpha$-KG has been shown in anesthetized dogs in a variety of states of acutely altered acid-base balance (61). Simultaneously measured A−RV differences from glucose were variable and not related to acid-base status, but net renal glucose production was essentially zero. This suggests that glucose utilization by the kidney balances ongoing gluconeogenesis. Presumably these two processes occur in different cells or in functionally different regions of the kidney. Support for this concept is available from studies of energy metabolism of renal tissue in vitro (21). However, renal glucose utilization by the dog kidney in vivo seems unlikely from the results of Chinard and co-workers (63). Tracer quantities of $^{14}$C-glucose were rapidly injected into a renal artery and an average of $97.6 \pm 10.4\%$ was recovered unchanged in the renal vein within 1 min. The scatter of values does not permit the conclusion that renal glucose uptake did not occur. However, recovery of $^{14}CO_2$ accounted for less than 0.2% of the injected glucose and only 1.6% of renal $CO_2$ production. Anaerobic glycolysis is not excluded by these results. Again, evidence obtained in the whole animal that glycolysis may not be energetically significant in vivo must be interpreted in light of the fact that such studies reflect primarily cortical metabolism. The metabolism of the renal medulla cannot be specifically distinguished from that of the cortex in vivo.

## METABOLISM AND FUNCTION OF RENAL MEDULLA

Renal cortex and medulla in vivo differ in at least four important ways: 1. There is a rich supply of mitochondria in cells of proximal and distal convoluted tubules of the cortex, whereas tubular cells within the medulla, comprising the loop of Henle and the collecting duct, contain few mitochondria (64). 2. Studies of the distribution of blood flow within the kidney have demonstrated that cortical flow is markedly higher than that of medulla (65, 66); the blood flow of cortex:

outer medulla:inner medulla is 472:132:17 ml/100 g/min (66). 3. Blood supply to the inner renal medulla is provided by vasa recta whose hairpin configuration permits operation of the counter-current multiplier by which a medullary osmotic gradient, necessary for the production of a concentrated urine, is generated (67). Thus, while cortical tissue is isosmotic with plasma, medullary tissue tonicity increases from the corticomedullary junction to the papillary tip. 4. The counter-current mechanism limits medullary oxygen tension (68, 69). Therefore, while cortical interstitium is well oxygenated, decreasing oxygen tension typifies the medulla from corticomedullary junction to the papillary tip.

As might be expected on the basis of these differences, the inner medulla displays a rate of oxygen consumption only 20–25% as high as an equal weight of cortex in vitro (20, 67). Conversely, the rate of anaerobic glycolysis in vitro is two (21) to three (20, 70) times higher in medulla, indicating the importance of this pathway to energy metabolism in this region. Glycolysis of both cortex and inner medulla has been observed to decrease in proportion to increasing oxygen tension ($pO_2$) in vitro (21), although in similar studies neither cortical (70) nor inner medullary (70, 71) lactate production (an index of anaerobic glycolysis) has been sensitive to changes in $pO_2$. In contrast, the specific oxidation of $^{14}C$-glucose to $^{14}CO_2$ by rabbit cortex in vitro is increased twelvefold as $pO_2$ is increased from 35 to 530 mm Hg, while the specific oxidation of glucose by inner medulla is relatively insensitive to this increase in available oxygen (70). Similarly, with glucose as substrate, the $Q_{O_2}$ of inner medulla increases relatively little (42%) as $pO_2$ is increased from 32 to 700 mm Hg (71). Thus, under aerobic conditions, the rate of glucose oxidation by medulla is only 30% of the cortical rate (70). The same pattern is seen with pyruvate as substrate (70).

A medullary dependence on glucose is also suggested by the presence of glycogen stores in medulla, which are 2–4 times the glycogen content of cortex (21, 72). Aerobic incubation of medullary slices without substrate results in rapid utilization of these glycogen stores. This can be prevented if glucose is provided, but pyruvate, palmitate, or other substrates have no such effect (21). Apparently there is an absence of enzymes in medulla needed for gluconeogenesis, specifically phosphoenolpyruvate carboxykinase, fructose 1,6-diphosphatase, and glucose 6-phosphatase (73). Therefore, gluconeogenesis does not occur there (21). Glycogen content of cortex is little affected by incubation without substrate, which suggests the availability of other endogenous substrates (e.g. lipids) (21, 44).

Although outer (red) medulla in vitro consumes oxygen at a rate approaching that of cortex (70, 74), the former tissue relies on glucose as its primary respiratory fuel (22, 70, 74). Moreover, the rate of anaerobic glycolysis by red medulla is the highest of any renal zone (70). The dominant pathway for glucose metabolism is oxygen dependent; by increasing $pO_2$ in vitro the rate of anaerobic glycolysis (lactate formation) falls very sharply, while glucose oxidation is concurrently enhanced (70). Outer medulla in vitro can also utilize palmitate as respiratory fuel. However, the presence of glucose reduces palmitate oxidation by 35% (22) while the reverse does not hold, in that the presence of palmitate does not affect the rate of outer medullary glucose oxidation (22). The behavior of renal cortex is just the opposite: addition of palmitate to cortical slices decreases glucose utilization (21, 75) while palmitate oxidation is unaffected by the presence of glucose (21). Moreover, under aerobic conditions with glucose and palmitate present, $CO_2$ produced by outer medulla is derived 43% from glucose and only 20% from palmitate while the remainder results from oxidation of endogenous substrates (22). Predominance of glucose utilization by outer as well as inner medulla is thus apparent.

The metabolic response of inner and outer medulla in vitro to increases in osmolality of the incubation medium indicates that medullary glycolysis may be resistant to the hypertonic environment characteristic of the renal medulla. Kean et al (20) found that anaerobic glycolysis by dog medulla increased threefold as the osmolality of the medium was increased from 300 to 1200 mOsm/kg with NaCl. Under the same conditions, cortical glycolysis was curtailed 50%. In comparable experiments, Bernanke & Epstein (71) have found a 60% inhibition of lactate formation by slices of dog inner medulla incubated in hypertonic NaCl (1200 mOsm). In both studies, however, aerobic metabolism of succinate was reduced as low as 56% of control by the hypertonic medium (20, 71).

Studies of outer medulla of rabbit indicate that glucose oxidation is doubled, while palmitate oxidation is unaffected as osmolality (as NaCl) is increased between 200 and 900 mOsm/kg (22). Aerobic lactate production from glucose is unaffected by the increased osmolality, but anaerobic lactate production from glucose decreases 50%. The data suggest that the stimulation of outer medullary glycolysis by hypertonic sodium chloride results from stimulation of sodium and potassium-activated adenosine triphosphatase (Na-K-ATPase). This enzyme system is considered in a subsequent section. Outer medullary respiration has been previously shown to increase in hypertonic media (76).

The importance of glycolysis to renal sodium transport has been suggested by the observation that iodoacetamide, a potent inhibitor of the glycolytic enzyme glyceraldehyde-phosphate dehydrogenase, is strongly diuretic when infused into the renal artery of a dog (26). This effect is confirmed by the similar studies of Martinez-Maldonado et al (77) who have found increases in sodium excretion along with decreases in free water clearance during renal arterial infusion of iodoacetamide in suitably prepared dogs. Since generation of free water is a function of the renal medulla, an important role for glycolysis in this process is implied. Similarly, micropuncture studies in the rat have revealed that iodoacetamide reduces sodium and water reabsorption in the distal nephron (30). Like most inhibitor compounds, iodoacetamide may inhibit more than one metabolic pathway, thus clouding interpretation. The effect of iodoacetamide on renal oxygen consumption has not been reported.

In parallel to the foregoing data, a body of evidence is accumulating that suggests significant energy production in renal medulla also results from lipid oxidation, a contention supported by studies both in vivo and in vitro. Slices of rabbit inner medulla oxidize palmitate 15–25% as rapidly as such slices oxidize glucose when both substrates are present aerobically (21). As Lee has pointed out (78), the complete oxidation of each mole of palmitate results in 130 mole ATP, while oxidation of 1 mole glucose produces 38 mole ATP. Thus, with sufficient oxygen, 40–50% of the theoretical ATP production by medulla could result from fatty acid oxidation. The oxygen requirement may be quite small, as suggested by the work of Bernanke & Epstein (71) previously described. They reported that in 5% oxygen, the theoretical production of ATP from succinate oxidation by dog inner medulla in vitro approximated that available from the utilization of glucose. In 100% oxygen, ATP theoretically available from medullary succinate oxidation was more than doubled and exceeded ATP available from glucose by 80%.

The presumed production of ATP by inner medulla from glucose appears to be constant as $pO_2$ is reduced because glycolysis increases as glucose oxidation decreases (70). With slices of outer medulla under aerobic conditions (95% $O_2$) oxidation of glucose accounts for only 18% of theoretical ATP production, while oxidation of palmitate and endogenous substrate account for 29 and 53% of available ATP (22) respectively, assuming the endogenous substrate is lipid (21, 44). Additional evidence of the importance of aerobic metabolism in renal medulla comes from the finding that cyanide decreases free water clearance and free water reabsorption in suitably prepared dogs (79, 80). The finding that cyanide as well as iodoacetamide diminishes the ability of the kidney to clear free water suggest that medulla, as well as cortex (see above) may have its energy needs met by the metabolism of a variety of substrates.

Finally, the effect of chronic potassium deficiency on renal slice oxygen consumption in the rabbit (81) and on mitochondrial enzyme content in rat kidney (82) has been investigated. Potassium deficiency produces a renal concentrating defect but does not impair renal diluting ability in vivo. The respiration of slices of outer medulla from potassium-deficient rabbits, in contrast to controls, does not increase upon addition of $K^+$ 5 m*M* to the incubation (81). Aerobic and anaerobic glycolysis by the slices is unaffected by potassium deficiency, as is cation-sensitive adenosine triphosphatase activity. A diminished activity of several oxidative enzymes in mitochondria is found in the outer medulla of potassium-deficient rats while enzyme activities of cortical mitochondria remain normal (82). These studies suggest that impaired oxidative metabolism may underlie the concentrating defect in hypokalemia and reinforce other documentation of the role of oxidative metabolism in this process.

## ADENOSINE TRIPHOSPHATE IN THE KIDNEY

If the metabolism of the renal cortex is like that of other tissues, energy provided by substrate oxidation is available for work through synthesis of the high energy compound ATP. It might therefore be expected that uncouplers of oxidative phosphorylation would inhibit renal sodium reabsorption by curtailing ATP formation. Curiously, this predicted event does not occur. The classic uncoupling compound 2,4-dinitrophenol (DNP) fails to effect a natriuresis in the dog undergoing saline diuresis, as originally demonstrated by Mudge & Taggart (83). This finding has subsequently been amply confirmed (9, 84). Although there is no exception to the lack of natriuretic action of DNP in the saline-loaded dog, one group has described a modest diuresis from DNP in dogs following water, mannitol, or glucose administration (85).

There is no question that DNP reaches renal mitochondria in vivo because its administration enhances renal oxygen consumption (9) and reduces cortical ATP content (86). This dissociation of oxidative metabolism from ion transport has led to active but unsuccessful attempts at explanation. DNP may inhibit sodium reabsorption when applied directly into the lumen of the proximal tubule (29) although even this observation is not universal (31). One explanation is that a part of the reabsorptive process may be energized directly by the electron transport system, bypassing ATP synthesis and degradation. However, specific data in support of this hypothesis have not been forthcoming.

Whether ATP is an indispensable source of energy for sodium transport in the kidney is important. Were ATP directly involved in energizing sodium reabsorption, inhibitors of oxidative metabolism or ATP synthesis that do block sodium transport should also reduce the turnover of ATP. This latter is not measurable in vivo with existing techniques. As a much less satisfactory alternative, the content of adenosine nucleotides in kidney tissue has been measured. ATP in the cortex of dog kidney, measured after rapid freezing in situ, is between 1.1 and 1.3 $\mu$mole/g wet weight (87). Because the half-life of cortical ATP is 3.3 sec (87), the values obtained are critically dependent on the rapidity of freezing. Following ischemia, the prompt decline in ATP is accompanied by a concomitant raise in AMP content with ADP remains constant. The reciprocal relationship between ATP and AMP content suggested that the following two reversible reactions are operative in cortex (88).

$$\text{ATP} \rightleftharpoons \text{ADP} + \text{Pi}$$

$$2\,\text{ADP} \underset{\text{Kinase}}{\overset{\text{Adenylate}}{\rightleftharpoons}} \text{ATP} + \text{AMP}$$

During ischemia these reactions are driven to the right. The presence of adenylate kinase appeared confirmed by the finding that $(\text{AMP})(\text{ATP})/(\text{ADP})^2 = 0.45$ in cortex after 9 sec of ischemia (88). This is close to the equilibrium value for the adenylate kinase reaction (89).

In contrast, the nucleotides of the dog kidney medulla have different characteristics. ATP is not sensitive to ischemia; its concentration remains constant after 60 sec of interruption of blood flow (90). Moreover, adenylate kinase appears to be absent from renal medulla, a finding consistent with the predominantly glycolytic character of this tissue (88).

Another approach in the investigation of the role of ATP in sodium reabsorption has been to administer inhibitor compounds known to interfere with ATP synthesis. Again, the main obstacle to this tactic is the technical impossibility of measuring ATP turnover in vivo. Only data on ATP content and incorporation of $^{32}$P into ATP have been reported. Cyanide, an inhibitor of cytochrome oxidase and sodium reabsorption, reduces renal ATP content (86) and uptake of $^{32}$P

(91). The natriuretic compound oligomycin, an inhibitor of phosphorylation, also decreases ATP content (86). However, the nonnatriuretic uncoupling agent DNP reduces both ATP content (86) and $^{32}P$ uptake (91). These observations constitute further indirect evidence dissociating sodium transport from ATP synthesis in the renal cortex.

The intravenous administration of glucose to dogs increases cortical ATP content nearly 50% (87). Under these circumstances cyanide fails to produce a decrease in cortical ATP and also in medullary ATP content, although the expected natriuresis still occurs. Similarly, infusion of DNP during glucose administration results in only a minor decrease in cortical ATP content as compared with its effect in the absence of glucose, and produces no decrease at all in ATP content of the medulla (87). These observations suggest that supplied glucose may be important to renal cortical energy metabolism and confirm the importance of glycolysis to energy production in the medulla.

## CATION-STIMULATED ADENOSINE TRIPHOSPHATASE

Like most cellular tissues, the kidney contains a cation-sensitive adenosine triphosphatase (Na-K-ATPase) characterized by 1. inseparability from the cell membrane (microsomal) fraction sedimenting at 105,000×g (92), 2. an absolute dependence upon $Mg^{2+}$ ion for activity, 3. further stimulation of activity in the presence of $Na^+$ and $K^+$, and 4. specificity for the $\gamma$-phosphate of ATP (93, 94). The activity of Na-K-ATPase in cellular tissues correlates closely with both the rate of a fraction of active cation transport (sodium for potassium exchange) and the rate of a fraction of cellular energy metabolism. These relationships have been reviewed in detail by Skou (95) and Katz & Epstein (92). The role of Na-K-ATPase in renal sodium transport has been carefully evaluated by Williamson (96) and more recently by Kiil (97) and Landon & Forte (6).

The Na-K-dependent portion of Na-K-ATPase activity is specifically inhibited by the cardiac glycoside ouabain (93, 94, 98, 99). In kidney tissue in vitro inhibition occurs at ouabain concentrations as low as $10^{-7}$ *M* and is complete near $10^{-4}$ *M* (98–100). A decrease in respiration of cortical homogenates occurs in proportion to the degree of Na-K-ATPase inhibition (98). In this same range of concentrations, oxygen consumption and active transport of sodium in exchange for potassium (Na/K exchange) are simultaneously inhibited in slices of kidney cortex (100). At a concentration of ouabain which inhibits Na-K-ATPase completely, respiration of kidney cortex slices and of homogenates is inhibited by 40% (98, 100), while Na/K exchange is abolished in the slices (100). Active transport of sodium in association with chloride and water (Na/Cl/$H_2O$ transport) is not, however, abolished by ouabain (101, 102), indicating the presence of two sodium transport systems in kidney tissue as suggested by earlier studies (103, 104). The role played by each of these systems in renal sodium reabsorption is undetermined. The simultaneous movement of sodium, chloride, and water is similar to the pattern of tubular reabsorption of much of the kidney, particularly the proximal tubule. The importance of this mode of sodium transport as a model for the renal reabsorption of sodium has been pointed out by Maude (105). It is further highlighted by the fact that Na/Cl/$H_2O$ transport in vitro is strongly inhibited by the potent diuretic ethacrynic acid which has only a modest inhibitory effect upon Na/K exchange (101, 102), but a somewhat greater effect upon Na-K-ATPase activity (94, 102, 106–108).

The importance of the Na-K-ATPase system to renal sodium transport in vivo has been inferred by the observation that injection of ouabain into one renal artery in the intact dog causes a marked ipsilateral natriuresis (12, 109, 110). The decrease in $R_{Na}$ is associated with a proportional decrease in $Q_{O_2}{}^{Ren}$ (12). With unilateral graded infusions of ouabain the degree of ipsilateral natriuresis correlates directly with the degree of ipsilateral inhibition of Na-K-ATPase in the whole kidney as determined after excision of the kidney (109).

Although the kidney may contain sufficient Na-K-ATPase to hydrolyze the ATP necessary for all sodium reabsorption (110), complete inhibition of Na-K-ATPase by ouabain in vivo results in the excretion of only 40% (not 100%) of filtered sodium (109, 111). This correlates well with the 40% depression of respiration by renal tissue in the presence of ouabain in vitro (98, 100). Clearly, other mechanisms are important, most likely those relating to Na/Cl/$H_2O$ transport (105).

The major anatomical site of action of ouabain within the kidney appears to be the thick ascending limb of the loop of Henle (112). This is in keeping with the intrarenal localization of Na-K-ATPase. The activity of the enzyme is higher in the outer medulla than in the cortex and is very low in the papilla (110, 113–115); the ascending limb of the loop of Henle is an outer medullary structure. Within the renal tubule Na-K-ATPase activity is greatest in the ascending limb, somewhat lower in the distal convoluted tubule, and considerably lower in the proximal convoluted tubule (115). Of further interest in regard to the function of the medulla, the Na-K-ATPase activity and oxygen consumption of outer medulla is stimulated by increased osmolality (NaCl or sucrose) while cortical Na-K-ATPase activity is not (116). The specific site of Na-K-ATPase within the cell is at the basal infolded membrane (117), the site of active sodium transport (1).

The functional importance of the Na-K-ATPase in the thick ascending limb may also be inferred by several observations in vivo related to the functions of the medulla (110, 112, 113, 118). Renal arterial ouabain infusion produces a striking decline in the metabolic rate of the outer medulla but only a modest decrease in that of cortex as measured by the heat accumulation technique (118). The renal arterial infusion of digoxin causes an ipsilateral decrease in free water clearance (119) and a decrease in free water reabsorption (113, 119) in vasopressin-treated dogs undergoing mannitol diuresis. In these animals the activity of Na-K-ATPase is reduced 50% in medulla and to a lesser extent in the cortex (113). In addition, the natriuretic effect of ouabain in dogs is augmented by saline loading (110), a maneuver which increases the delivery of sodium to the loop of Henle (120). The increased effect of ouabain is easily explained if sodium reabsorption at the loop of Henle is regulated by Na-K-ATPase (97). It is relevant in this context that medullary Na-K-ATPase activity is itself enhanced by saline loading (121).

Renal sodium reabsorption and Na-K-ATPase activity do not always appear interrelated, however. During unilateral renal arterial ouabain infusion in vivo a progressive decline in the Na-K-ATPase activity of the contralateral kidney was observed by Nechay & Nelson (111). No contralateral natriuresis occurred, while ipsilateral natriuresis would invariably occur when Na-K-ATPase activity was comparably depressed. This may indicate adaptive compensation by other mechanisms for sodium reabsorption on the contralateral side, for example, more avid reabsorption by proximal tubule or increased functioning of the Na/Cl/$H_2O$ transport mechanism. Nechay and co-workers (107) reported earlier that the nondiuretic mercurial agent *p*-chloromercuribenzoate (*p*-CMB) inhibits dog kidney Na-K-ATPase in vitro as strongly as do the mercurial diuretics chlormerodrin and meralluride. In addition, after in vivo administration *p*-CMB binds to the Na-K-ATPase-bearing plasma membrane fraction of dog kidney to the same extent as these diuretic agents. Although none of these agents appears to inhibit renal Na-K-ATPase significantly after in vivo administration to dogs, chlormerodrin diuresis is not further augmented by ouabain, whereas *p*-CMB does not block ouabain diuresis (107). This suggests that chlormerodrin in vivo may gain access to a critical transport-linked Na-K-ATPase site in a manner analogous to ouabain while *p*-CMB does not.

The cellular mechanisms underlying the actions of diuretic agents have been reviewed by Landon & Fitzpatrick (122) and Landon & Forte (6). In rat kidney cortex meralluride inhibits Na-K-ATPase in vitro and in vivo. It also blocks Na-K-ATPase-dependent cortical respiration in vitro and membrane Na-K-ATPase-dependent stimulation of cortical cell sap glycolysis after administration in vivo (122). In contrast, *p*-CMB inhibits neither the enzyme activity nor the metabolic systems. The clue to these differences may reside in the finding that the binding of chlormerodrin to protein subfractions of the Na-K-ATPase-rich membrane differs from that of *p*-CMB (122). The failure of ethacrynic acid to evoke diuresis in the rat, despite inhibition of Na-K-ATPase (106), may have a similar explanation, since the binding of ethacrynic acid to the plasma membrane subfractions differs from that of either chlormerodrin or *p*-CMB (122). Such findings appear to indicate that the function of the Na-K-ATPase system depends upon site-specific relationships to membrane structure.

Other discrepancies between renal sodium reabsorption and renal Na-K-ATPase activity have been reviewed by Williamson (96). After unilateral nephrectomy the increase in $R_{\mathrm{Na}}$/unit kidney weight by the remaining kidney is paralleled by an increase in renal Na-K-ATPase activity/mg microsomal protein, in the absence of increased specific activity of other microsomal enzymes (123). However, increase in $R_{\mathrm{Na}}$ has been found to precede the increase in Na-K-ATPase activity (124) or to occur in its absence (125). Adrenalectomy results in sodium wasting and decreased specific activity of Na-K-ATPase (123) but changes in plasma electrolytes may precede changes in enzyme activity (126). Methyl prednisolone or protein loading increases ATPase activity (123). The increase with methyl prednisolone is the result of increased plasma membrane per cell (127). Neither mineralocorticoids (128) nor a low sodium diet (129), which decreases renal sodium excretion, increase the specific activity of Na-K-ATPase.

Both glycolytic and oxidative pathways are stimulated by Na-K-ATPase in vitro (130–132). The addition of the Na-K-ATPase-rich microsomal membrane fraction of rat kidney cortex markedly stimulates the glycolytic rate of the cell cytoplasm in vitro (130). The site of stimulation has been localized to the reaction catalyzed by phosphoglycerate kinase (PGK):

$$\text{1,3-diphosphoglycerate} + \text{ADP} \underset{\text{Mg}^{2+},\ \text{Pi}}{\overset{\text{PGK}}{\rightleftharpoons}} \text{3-phosphoglycerate} + \text{ATP}$$

The stimulation is not eliminated by adding excess ADP to the system, and ATP can be substituted for ADP without decreasing the glycolytic rate, implying that free ADP is not generated. Presumably ATP produced by the reaction is hydrolyzed by the Na-K-ATPase and reutilized in the reaction. Comparable results have been reported by others (131). An important role for glycolysis in renal sodium reabsorption is suggested by these findings. The membrane Na-K-ATPase also stimulates mitochondrial respiration (131). This may be via direct release of ADP (122). In addition, stimulation of glycolysis would result in free ADP from hexose phosphorylation; this ADP might then be available to stimulate mitochondrial respiration.

The infusion of ouabain into one renal artery of fasted, saline-loaded dogs, though decreasing $R_{\text{Na}}$, has no effect upon cortical ATP content (87, 88). Since inhibition of Na-K-ATPase might be expected to result in a rise of tissue ATP content, this finding (also) suggests a limited role for Na-K-ATPase in cortical (proximal tubular) sodium reabsorption. However, renal arterial ouabain infusion does result in increased cortical ATP content in saline-expanded dogs receiving intravenous glucose, as compared to controls not receiving ouabain, and the same effect obtains in the medulla (87). This suggests indirectly that glucose may facilitate cortical Na-K-ATPase activity, an hypothesis consistent with the interaction between Na-K-ATPase and glycolysis (130). The elevation of medullary ATP content after ouabain infusion may be explained by the higher activity of Na-K-ATPase in medulla (110, 113–115) and the apparent importance of its role in normal medullary function (110, 112, 113, 118).

## SUMMARY

In summary, Na-K-ATPase, though present in cortex, is most active in the renal outer medulla and appears to play a role in approximately 40% of renal sodium reabsorption. The mechanism of this fraction of sodium reabsorption is likely to be Na/K exchange. Although Na-K-ATPase-dependent renal sodium reabsorption is associated with proportional renal oxygen consumption, a specific relationship between Na-K-ATPase and glycolysis has been suggested. These findings are compatible with the metabolic capabilities, both oxidative and glycolytic, of the renal outer medulla. The failure of some inhibitors of Na-K-ATPase to evoke a diuresis under some circumstances in vivo may find explanation in physiological compensation by Na-K-ATPase-*independent* sodium transport mechanisms, or by requirements for site specific interaction of inhibitor with Na-K-ATPase-dependent transport systems which are not fulfilled in vivo.

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# CLINICAL PHARMACOLOGY OF DRUG INTERACTIONS[1]

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## INTRODUCTION

Appropriate drug therapy improves the quality of life while improper use may create iatrogenic disease. In an age where an individual patient may receive eight or more prescribed drugs and self-medicate himself with more, the stage is set for a veritable Pandora's Box of adverse reactions. Countless reports and several books on possible deleterious drug interactions (1–3) have contributed to a peculiar mystique which generates anxiety but does not always provide suitable therapeutic alternatives. In order that the desired therapeutic effect be achieved, insight into the mechanisms by which drugs act and interact is needed, especially in those areas where inappropriate use of drugs negates the desired response. Findings in human subjects are emphasized. The reader is also referred to several recent excellent reviews of drug interactions (4–8).

## WHAT ARE DRUG INTERACTIONS?

The term drug interaction usually refers to an adverse effect produced by two drugs meeting in a patient which may be unsuspected from the action of either agent alone. Drugs interact at sites of absorption, binding, transport, and metabolism, and hopefully at the receptor site, where the desired therapeutic effect is elicited. Drugs may alter a host of seemingly unrelated metabolic processes and profoundly influence unborn generations. A particular disease state or the genetic constitution of the patient may dramatically modify the expected response.

## MECHANISMS OF DRUG INTERACTIONS[2]

*In vitro.*—Even before drugs reach the patient, the mischief begins if incompatible drugs are mixed together. For instance, acidic and basic drugs tend to neutralize each other. This is beneficial when basic protamine counteracts heparin in vivo, but many other compounds will do this if placed into the same

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[2] See Table 1.

TABLE 1. Drug Interaction Mechanisms

- In Vitro
  - Inactivation
  - Incompatibility
- Mode of Administration
  - Route
  - Speed
  - Metabolism
  - Gastrointestinal
    - Solubility
    - Interaction with other agents
    - Alteration in flora
    - Interaction with body constituents
- Plasma Albumin
  - Competitive
  - Noncompetitive
  - Metabolite competition
- Receptor Site
- Nonspecific Binding
- Hepatic Transport Proteins
- Biotransformation
  - Phase One-microsomal metabolism
    - Induction-accelerated metabolism
    - Inhibition
      - Competitive
      - Noncompetitive
- Hepatic Excretory Transport Proteins
- Biliary Excretion
  - Competition
  - Bile salt metabolism
- Pharmacologic Effect
  - Interaction expected if pharmacologic action understood
- Renal Excretion
  - Filtration
    - Protein binding
  - Reabsorption
    - pH
    - Liposolubility
  - Tubular secretion
    - Competition for specific pathways
- In Vitro
  - Alteration of laboratory tests
- Genetic Predisposition
- Modification of Drug Handling by Disease
- Diseases Due to Drugs

intravenous solution (9). Gentamicin is inactivated when combined with carbenicillin or ampicillin in the same infusion, but this does not occur significantly in serum. Patients should receive these drugs by separate routes (10). Glucocorticoids, commonly combined with antimicrobials in topical therapy, may interact with antimicrobials such as fusidic acid and hamycin to decrease the antibacterial effect (11). Plastic receptacles may present other problems by allowing materials to seep into infusions or by failing to provide appropriate storage conditions for drugs such as nitroglycerine, which must be stored in glass (12, 13).

*Route of administration.*—The same drug may yield distinct pharmacologic effects if ingested, applied topically, or administered intravenously. For most drugs the gut behaves as a lipid membrane, receiving the un-ionized liposoluble form of drug most effectively. The dosage form may affect the absorption and has recently been well summarized (14, 15). Drugs which alter intraluminal pH, interact chemically, or affect gastrointestinal transit time may interfere with the absorption of other agents. Phenobarbital impairs the absorption of griseofulvin by an unknown mechanism but does not enhance hepatic metabolism (16). Many drugs are highly bound by the liver on the first passage through this organ from the gut, so that optimal concentrations are not attained in blood and at receptor sites until the liver is saturated. Proportionally less drug may be cleared by the liver on the first passage after parenteral administration of the same drug. Extrahepatic tissues, including the gut, can produce organ-specific metabolites with distinct pharmacologic or toxic properties.

*Serum protein binding.*—Whether injected or ingested, most drugs are ultimately distributed to the body through the blood bound to plasma proteins to some extent. Only the free drug, in equilibrium with the bound drug, is at liberty to equilibrate with sites of action, inactivation, and storage. Drugs may bind to a variety of plasma proteins, particularly albumin, which is generally assumed to have a variety of binding sites, but one major binding site for each drug or endogenous substrate. Drugs, metabolites, and known (bilirubin) or unknown (uremia) endogenous substances compete directly for specific binding sites, or indirectly by imposing an altered conformation that impedes attachment of substances elsewhere on the molecule. Since only the free drug is in equilibrium with sites of action and metabolism, a small displacement of a highly bound drug increases the free drug concentration and thus enhances the pharmacologic effect. Serum proteins act as inactive reservoirs for extensively bound drugs, but blood level determinations do not always discriminate between free and bound drug. Certainly in disease states where uremia, hyperbilirubinemia, or hypoalbuminemia is manifest, the total concentration of a drug may be low or normal, but if a greater proportion is free, pharmacologic or toxic effects are exaggerated (17). The concentration of free diphenylhydantoin is similar in cerebrospinal fluid and plasma when measured under steady-state conditions in patients on no other therapy (18).

Amounts of binding protein are an important determinant of drug stores and accessibility to sites of elimination and action, and may also correlate with short- and long-term side effects. Lewis and his co-workers in the Boston Collaborative Drug Surveillance Program observed that the incidence of prednisone side effects doubled in patients with serum albumin levels lower than 2.5 g (19). Prednisone and its metabolite prednisolone are bound to cortisol-binding globulin, but the capacity of this carrier is exceeded with chronic administration, and the excess drug is carried partly by serum albumin. Optimal prednisone dose schedules for patients with low serum albumin need to be formulated.

While most drugs and endogenous substrates bind reversibly to serum proteins, the transfer of the acetyl group from acetylsalicylic acid may permanently alter the protein (20). In vitro studies suggest acetylation may alter binding in both directions: the affinity of human serum albumin increases for phenylbutazone but decreases for flufenamic acid (21). Since aspirin consumption is now ubiquitous (22), this presents a whole new ballgame for drug binding studies. Metabolites of both chloral hydrate and warfarin seem capable of displacing drugs bound to albumin. The role of specific metabolites as determinants of protein binding requires further study.

*Receptors.*—Receptors have high affinity but low capacity for pharmacologic agents and are the sites where the drug "does its thing" (24), bound in minute concentrations. In general, the greatest proportion of a drug in the body is at sites other than its active one. The speed of administration determines the efficacy of treatment with diazoxide, an antihypertensive drug that must be administered rapidly intravenously in order to saturate receptor sites rather than sites of inactive binding (23). This is in contrast to the recommendations for intravenous administration of most other drugs, which tend to elicit potent therapeutic and toxic effects if injected too rapidly.

*Biotransformation.*—Biotransformation of many therapeutic agents occurs in the liver. Two hepatic organic anion binding proteins that transport some drugs and organic anions to intracellular sites of metabolism have been characterized (25). Competition by drugs, drug metabolites, and endogenous substrates for sites on such proteins seems likely. During the first stage of drug metabolism the drug is transformed by microsomal or soluble enzymes to metabolites, which may be more, the same, or less active pharmacologically than the parent compound and possess different pharmacologic and toxic properties. In a second phase of drug metabolism, conjugation reactions form water-soluble metabolites that are readily excreted.

The microsomal system, which resides in the endoplasmic recticulum, affects biologic oxidations and hydroxylations via hemeprotein P-450 and its electron transport chain. The presence of many substrates may induce increased amounts of enzyme and expands the capacity for drug metabolism. This system is rather unusual as it is flexible enough to transform hundreds of substrates, including endogenous steroids and hormones, by a mechanism not yet fully understood. This versatility may be nature's provision for the body to detoxify compounds it has never seen before. The P-450 enzyme system is present in tissues other than liver. In the adrenal, ovary, and probably placenta these enzymes are involved with steroid hydroxylations, but in lung, kidney cortex, and skin the role is less well defined (26). There is recent evidence for the presence of a similar hydroxylating system in human lymphocytes (27). A recent report stresses the possible importance of metabolites in certain idiosyncratic toxic reactions. An immune thrombocytopenia was produced not by the analgesic N-acetyl-*p*-aminophenol, but by antibodies which developed to the sulfate conjugate of the drug (28). Metabolites of other agents will undoubtedly be found to have antigenicity and explain some other adverse drug reactions, currently lumped in the category of hypersensitivity reactions. Toxic reactions affecting lung, kidney, skin, blood constituents, and liver are likely to result from the peculiarities of metabolites produced locally, or elsewhere, that have specific effects on such tissues, by both immune and nonimmune mechanisms. The number of specialized systems that exist for the elimination of drugs and their respective metabolites from the liver is unknown, but at least some types of adverse hepatic reactions result from drug interference with bile salt secretion (29, 30).

*Pharmacologic action.*—Some interactions between drugs can be avoided simply by remembering the pharmacologic action of the drug. A recent clinicopathological conference in an erudite medical journal ascribed the death of a patient to his disease, but an alert clinical pharmacologist noted that this was an example of drug interaction. The patient received azathioprine, which is ultimately inactivated by the enzyme xanthine oxidase, as well as allopurinol, which inhibits xanthine oxidase. Pronounced hematologic toxicity resulted from excessive azathioprine (31).

*Elimination.*—In contrast to the gut, the kidney behaves as a barrier to lipids. Only the free, non-protein-bound drug is filtered at the glomerulus. Changes in pH that affect the partition of ionized and un-ionized drug in the urine will affect tubular reabsorption, and reabsorption of un-ionized drug will be favored. The plasma half-life of pseudoephedrine can be markedly decreased by acidification of urine and increased by alkalinization (32). Classes of drugs share pathways for tubular secretion and may compete with each other. Probenecid inhibits the renal tubular secretion of sulfinpyrazone as well as the *p*-hydroxy metabolite of sulfinpyrazone (33).

Kidneys are an important route of elimination for drugs not extensively inactivated elsewhere in the body. Therefore, a patient with renal disease will require a greater dose adjustment if given a drug that is largely excreted unchanged than if he receives a drug that is extensively transformed to inactive metabolites prior to renal elimination.

*Pharmacogenetics.*—Exposure to environmental factors, which include drugs, may alter man's complex genetic capacity for drug metabolism. Human pharmacogenetic studies have compared the handling of drugs in pairs of identical and fraternal twins as well as unrelated individuals. Classic studies by Vesell & Page indicated that the capacity to metabolize antipyrine and dicumarol is genetically controlled. Studies with phenylbutazone (34) and nortriptyline (35) suggest that a number of genes may be involved, which is not surprising since a number of enzymes are also involved. While a number of drugs may be transformed by the same or similar microsomal systems, a patient may handle different substrates at varying rates. Inducer drugs may increase the microsomal capacity for only some drugs handled by this system. For example, treatment with halofenate, a new hypolipidemic agent, may accelerate disposition of antipyrine and bishydroxycoumarin but retard metabolism of warfarin (36). Fast metabolizers of glutethimide handle amobarbital rapidly, but this is unrelated to the biotransformation of antipyrine (37). All of six volunteers chronically treated with ethanol accelerated their elimination of antipyrine; in three patients the half-life of ethanol was also shortened but in the three others ethanol disposition became prolonged (38).

A nonmicrosomal system that has been well studied genetically is the acetylation system, which transforms isoniazid, sulfamethazine, phenelzine, hydralazine, and dapsone (39). In the United States, the population seems evenly divided between slow and fast acetylators of isoniazid, but this varies in different parts of the world. Slow acetylators tend to have higher blood levels on usual doses and a higher incidence of adverse drug reactions as well as drug interactions. The acetylation capacity is not known to be inducible. While studies are progressing to assess the role of genetics in microsomal and conjugation reactions, little is known of genetic factors that may affect serum protein binding, drug distribution, or pathways of elimination.

In addition to therapeutic agents, many environmental factors such as exogenous and endogenous atmospheric pollutants may alter drug handling capacities. Workers chronically exposed to chlorinated hydrocarbon insecticides have an enhanced capacity for antipyrine metabolism. Conversely, insecticide residues tend to be diminished in patients chronically medicated for seizure disorders (40). Cigarette smokers have decreased plasma concentrations of phenacetin, possibly resulting from enhanced metabolism (41), and pregnant smokers have lower serum vitamin $B_{12}$ levels than similarly gravid nonsmokers (42). Patients who smoke or reside in highly polluted environments require higher maintenance doses of pentazocine used to supplement nitrous oxide anesthesia (43).

At the extremes of life, the capacity for metabolism of a number of drugs is low. Recent studies in elderly individuals suggest decreased ability to dispose of antipyrine and phenylbutazone, most striking in elderly women (44).

Reviews of drug effects on vitamins (45) and the implications of enzyme induction on other body constituents are recommended for further reading. (46)

## DRUG LEVELS

Since measurements are available for limited numbers of drugs, their full value will become apparent with time. The complexities of drug absorption, distribution, and metabolism have indicated that for many agents the amount of drug available for pharmacologic effect bears no relation to dosage given but is often related to the amount present in the blood. Even blood level data must be interpreted with many reservations. Assays must be sensitive and specific, and it should be clear whether parent drug, and/or active or inactive metabolites are included in the result. For drugs that are highly protein bound, expression as total drug in blood is far less meaningful than partition into free and protein-bound forms. In what sort of space does the drug normally distribute and how is it likely to distribute in the particular patient? While drug level determinations may be exceedingly helpful, interpretation must be considered in clinical context, like other laboratory tests. The usefulness of serum drug concentrations has recently been reviewed (46a). In the sections that follow, drug level measurements are discussed in relation to clinical usefulness and drug interactions.

### ANALGESICS—SALICYLATES

Although the medicinal benefits of salicylates have been enjoyed for centuries, their pharmacokinetics have only recently been elucidated (47). The pharmacologic effect of these agents increases markedly with increasing dosage because salicylates are inactivated by systems of limited capacity that are readily saturated at ordinary clinical doses. Such limited metabolic capability explains why the elimination half-time of a given dose of salicylate increases from 3 to more than 20 hr if the dose is also increased, a reaction especially apparent in intoxication (48).

Levy and his co-workers have carefully established the pharmacokinetic implications of each of the pathways of salicylate metabolism (49, 50). A dose of aspirin is rapidly hydrolyzed to salicylic acid, which has a half-life of 3 to 4 hr. Salicylic acid, in turn, is eliminated partly unchanged by renal excretion and partly by metabolic conjugations. Conjugation of salicylic acid with glycine forms salicyluric acid. Other metabolites are acyl and phenolic glucuronides, and a minor portion is converted to gentisic acid. In man, the capacities for glycine conjugation and phenolic glucuronidation are limited (51), but formation of salicylate, acyl glucuronide, and gentisate are independent of the concentration of drug in the body (49, 50).

The conversion of salicylate to salicylurate has recently been characterized in bovine hepatic "mitochondria," and consists of two major reactions. Salicylate is activated enzymatically to salicyl-CoA, which then combines with glycine (52, 53). The first or salicyl-CoA synthetase activity is rate-limiting under optional in vitro conditions.

Sulfation and glucuronidation of salicylamide is also readily saturated but, in contrast to glycine conjugation, sulfate formation can be increased if sulfate donor compounds are supplied (51). Salicylic acid and salicylamide compete for a common glucuronidation pathway (50). While benzoate and salicylate are both conjugated with glycine, different steps may be rate-limiting and there may be species differences in the number of enzymes involved (50, 52). Such competition may diminish elimination of both drugs or interfere with the elimination of other drugs concomitantly administered that depend on limited capacity systems for removal. Salicylamide and acetaminophen compete for formation of sulfate and glucuronide metabolites. Such competition might be beneficial if used to develop more effective oral therapy for drugs ordinarily subject to inactivation by intestinal mucosal conjugations and first pass hepatic biotransformation (50).

Salicylates bind to plasma proteins, competing with endogenous substrates such as thyroid hormones, bilirubin, and urates. Competition and displacement of penicillin, sulfinpyrazone, and methotrexate may increase the free drug and thus potentiate toxicity.

A change of less than 1 pH unit downward in the urine of a patient receiving therapeutic doses may facilitate renal tubular reabsorption of salicylate, so that the serum level doubles and toxicity results (54). Urinary alkalinization promotes salicylic acid excretion and is especially useful in intoxication, when the limited metabolic systems have long been saturated and salicylic acid is the predominant metabolite. Salicylurate formation was the same during accidental intoxication and after recovery (one tenth of the amount was administered), dramatically illustrating the limitation of this system.

Although the first use of an artificial kidney in the United States occurred in the treatment of salicylate intoxication, the quest for better treatment methods continues. Activated charcoal, rediscovered, is very effective in preventing salicylate absorption if the drug is still present in the gastrointestinal tract after ingestion (55). Addition of serum albumin to peritoneal dialysate fluid doubles the amount of salicylate removed by this procedure. In salicylate-intoxicated animals, brain glucose levels were abnormal even when blood glucose levels were normal, and survival was increased by glucose administration (56). Serum levels of salicylates are helpful in assessing the severity of intoxications (57, 58) but levels obtained early after ingestion may be misleading if more drug is subsequently absorbed.

Aspirin and acetaminophen combined have a greater antipyretic effect than either agent alone, without affecting serum levels of aspirin (58a). The mechanism of this interaction is uncertain, and studies are in progress to evaluate the suitability of this combination for sustained therapy. In rats, pretreatment with microsomal inducers such as phenobarbital and phenylbutazone decreases in vitro hepatic hydrolysis of aspirin (59), but in man phenacetin pretreatment has no such effect (60).

Among normal individuals, genetically slow and rapid metabolizers of salicylates have been described (48). Down's syndrome (trisomy G) patients excrete more free salicylic acid and less salicyluric acid than normal subjects. The distribution and metabolism of acetylsalicylic acid is altered, which may be partially attributed to diminished glycine conjugation (61). Patients with rheumatoid arthritis have low plasma ascorbic acid levels, but after treatment with antiinflammatory agents, including aspirin, platelet ascorbic acid is reduced as well (62).

Claims that an aspirin-indomethacin interaction is mediated by alteration of serum indomethacin concentrations have not been supported by studies in man using a spectrofluorometric assay for indomethacin (63). However, aspirin ingestion produces fluorescent substances in urine that interfere with the fluorometric measurement of urinary 5-hydroxyindoleacetic acid (64). Aspirin and indomethacin decrease the urinary output of D-xylose (65). Indomethacin, neomycin, and phenformin decrease intestinal absorption of D-xylose. Aspirin diminishes xylose excretion even when xylose is administered parenterally, which suggests a site of interaction other than absorption. A therapeutic dose of aspirin also decreases the plasma clearance of $^{125}$I-diatrizoate (Hypaque) (66). It is not known whether this reflects an effect of aspirin on renal function or an interaction between aspirin and Hypaque.

## ANTICOAGULANTS

Since anticoagulant drugs are not infrequently "a cause for interring the bones of an unfortunate patient who suffers a catastrophic bleed," rational standards for safe therapy must be established and heeded (80). Efficacy of the anticoagulant drugs remains controversial and will not be discussed.

*Heparin.*—Two recent reports indicate a substantial risk of major bleeding in elderly women receiving heparin (67, 68). Congestive heart failure may be a predisposing factor, but potential risk factors such as decreased serum protein levels, administration of drugs that alter clotting such as aspirin, and relation of dosage to body weight are not specifically mentioned. Such reports emphasize the need for more information on heparin kinetics and interactions.

The avid binding of heparin to many proteins may impair the reproducibility of existing plasma assays.[3] Studies of heparin kinetics have largely depended on its biologic, anticoagulant effect. The mean half-life of anticoagulant effect is 1.5 hr. Pharmacokinetic calculations suggest that the most practical and constant anticoagulant effect would be achieved by continuous intravenous infusion of low doses (70). This has not been tested in a controlled, prospective trial, and doses lower than those commonly used may be effective and warrant study. Estes has determined that the activated partial thromboplastin time (APTT) provides the greatest precision in monitoring the half-life of anticoagulant activity (70), while Basu and co-workers found the APTT of value in regulating therapy to prevent recurrent thrombosis (71). This test did not identify potential bleeders. Heparin given as continuous intravenous infusions in sorbitol or dextrose did not appear to have impaired potency in patients (72), in contrast to earlier reports, although fairly high doses were used in this study.

*Coumarin anticoagulants.*—Perhaps the best known and most widely publicized drug interactions have concerned the oral anticoagulants. Several eloquent, extensive reviews have recently appeared and are strongly recommended (24, 73, 74).

Warfarin impairs clotting factor synthesis, but the exact mechanism is still being unraveled. Studies of patients and animals genetically resistant to the effects of warfarin suggest that warfarin and vitamin K may act as competitive antagonists at a common receptor site (75). Warfarin binds to microsomal membranes and this binding capacity is decreased in warfarin-resistant rats (76). Alternatively, warfarin may exert its anticoagulant action by an effect on vitamin K metabolism. Recently, phylloquinone oxide (phylloquinone-2,3-epoxide) has been identified as a metabolite of vitamin $K_1$ in rats (77). The conversion of vitamin $K_1$ (phylloquinone) and the reverse reaction occur extensively in normal animals, but warfarin treatment blocks the conversion of the oxide back to vitamin $K_1$. Does warfarin act by inhibiting the reduction of the oxide back to vitamin $K_1$? In hereditary warfarin resistance, then, a genetically altered reductase enzyme might be less sensitive to inhibition by warfarin. This intriguing concept requires further exploration.

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[3] Perhaps this binding affinity might be exploited by developing techniques based on the affinity of this drug to bind to resins or by using chromatographic methods which employ inert supports such as glass beads (69).

The coumarin anticoagulants tend to be un-ionized at normal gastric pH and thus absorbed in the stomach and upper gastrointestinal tract. They are bound extensively to plasma proteins, particularly albumin. Only the unbound drug, which comprises a small percentage of total drug in plasma, is free to equilibrate with sites of action, biotransformation, and excretion. Serum albumin has two binding sites for each of the coumarin drugs and these sites can be influenced by the environment (78). In the absence of other therapy, individuals do not appear to differ appreciably in the extent to which these drugs are bound to plasma albumin. Since amounts of free drug are small, displacement of small quantities of protein-bound drug may result in dramatic increases in the concentration of free drug in plasma, which leads to a pronounced pharmacologic effect. Extensive albumin binding provides a continuing source of free drug, and explains the long plasma half-lives observed. Average plasma half-lives are 2.5 hr for ethylbiscoumacetate, 1 day for acenocoumarol and bishydroxycoumarin, 2 days for warfarin sodium, and 6 days for phenprocoumarin. Individual differences in the half-lives of these drugs reflect differences in the rates of hepatic microsomal metabolism, which may be influenced by genetic and environmental factors. All the metabolites of the oral anticoagulants have not been identified in man. Extensive biotransformation produces water-soluble metabolites readily excreted by the kidney. Most of the recognized metabolites possess no clinically significant anticoagulant activity, but further work in man is needed (79).

A metabolite of warfarin, 5-OH warfarin, has no anticoagulant activity but has greater albumin binding affinity than the parent drug (78) and studies are needed to see if this metabolite displaces the parent drug under physiological conditions (79).

The half-lives of the monocoumarins (warfarin, acenocoumarin, phenocoumarin) are independent of their plasma concentrations, while the half-lives of the dicoumarins (bishydroxycoumarin and biscoumacetate) increase with increasing plasma concentrations. This may be due to saturation of hepatic metabolism. A no-load technique in which patients are begun on maintenance rather than loading doses of warfarin may avoid precipitous clotting factor depression in an unsuspected slow metabolizer (80). These drugs do not appear to induce hepatic microsomal biotransformation.

The anticoagulant action of the coumarins may be altered by interference with the bioavailability of vitamin K, modification of hemostasis, or by an effect on coumarin absorption, binding to albumin, hepatic biotransformation, or receptor affinity. Calculation of the ratio of prothrombin time prolongation to either the coumarin dose (coumarin dose index or CDI) (24) or the coumarin plasma concentration (coumarin concentration index or CCI) (24) has been useful in discerning whether a significant drug interaction appears likely when drug therapy is changed. Because of marked species variation in binding and biotransformation, extrapolation of results of animal studies to man are unreliable, and evaluation of possible interactions with anticoagulant drugs requires study in patients under clinical conditions. Improper filling of blood drawing tubes may falsely elevate prothrombin times and lead to much confusion (81).

Many drugs have been reported to interact with the oral anticoagulants. Because of individual variation, the effects of any combination known to cause alteration of anticoagulation cannot be predicted in a given patient, and there is no substitution for careful monitoring when either therapy or the patient's condition changes. Remember that the effects of a change in therapy may not be apparent for several days or weeks. The new steady state will depend on the half-lives of the anticoagulant and the second drug, the nature of the interaction, and the rate of biotransformation in the individual patient. It may be many days or weeks before a new steady state is achieved when two drugs with long half-lives are suddenly administered together. This is particularly true when hepatic microsomal inducers, which result in accelerated coumarin degradation, are used. On the other hand, the effects of a drug that interacts predominantly by displacing coumarin from plasma albumin binding sites are likely to be acute, occurring within the first few hours or days.

Drugs that significantly decrease the hypoprothrombinemic action of coumarins by inducing liver enzymes in man include all the barbiturates and glutethimide. Ethchlorvynol (Placidyl) antagonizes the hypoprothrombinemic effect, but the mechanism is not established. Estrogens and adrenocortical steroids may increase anticoagulant requirements by increasing clotting factor synthesis. Both ascorbic acid and methyl polysiloxane, an additive in cooking oil, may decrease anticoagulant effect and require study. If a patient has been chronically anticoagulated and one of the drugs that decrease anticoagulant effect is added, the patient may require more anticoagulant to maintain the same prothrombin response. If a patient is chronically receiving a drug that decreases the hypoprothrombinemic action of coumarin and needs anticoagulation, he may require a greater than average maintenance dose of coumarin. If a patient is receiving coumarin with a drug that decreases the hypoprothrombinemic action, and the second drug is suddenly discontinued, the coumarin dose should be adjusted downward to avoid undue bleeding.

The hypoprothrombinemic effect of coumarins is significantly potentiated by *n*-alkyl-substituted anabolic steroids, chlortetracycline and neomycin, mercaptopurine, clofibrate, dextrothyroxine, and glucagon, but the mechanism of interaction is unknown. Allopurinol, chloramphenicol, disulfiram, and probably nortriptyline and phenyramidol increase the hypoprothrombinemic effect, probably by interfering with hepatic inactivation. Studies in dogs indicate that isoniazid and paraminosalicylic acid (PAS) potentiate bishydroxycoumarin anticoagulation (82). The mechanism is unknown, and interference with hepatic metabolism is suggested. Interactions between anticoagulants and antituberculous drugs need to be investigated. Inhibition of ethyl biscoumacetate metabolism by methylphenidate has been reported but has not been confirmed (83). Drugs, especially acids highly bound to albumin and administered in large doses, are likely to displace anticoagulants from sites on plasma albumin. Mefenamic acid substantially prolongs the prothrombin time when administered to anticoagulated patients. Nalidixic acid, diazoxide, and ethacrynic acid displace warfarin from human albumin when studied in vitro, and the clinical significance must be assessed. Phenylbutazone, oxyphenbutazone, indomethacin, sulfinpyrazone, and long-acting sulfonamides are potent displacers of albumin-bound coumarins in vitro or in experimental animals. This effect would be expected to occur in man. Phenylbutazone may competitively displace warfarin from plasma and hepatic binding sites, increasing the availability of the drug to the pharmacologic receptor, then allowing enhanced warfarin degradation by microsomal induction.

A major hepatic metabolite of chloral hydrate, trichloroacetic acid, accumulates and displaces warfarin from albumin during chloral hydrate therapy (24). Transient hypoprothrombinemia may result if chloral hydrate therapy is initated in patients on chronic warfarin treatment. Patients receiving continuous chloral hydrate therapy during the first four days of warfarin anticoagulation required less warfarin than those receiving no chloral hydrate (84), but thereafter warfarin requirements in both groups were similar. Clinical studies of patients receiving concomitant warfarin and chloral hydrate for long periods of time also find no interaction after the initial phase of anticoagulation (85). Chloral hydrate does not decrease the hypoprothrombinemic action of coumarins in man, as was previously supposed. Like phenylbutazone, the shortened warfarin half-life with chloral hydrate reflects enhanced availability of warfarin for the receptor.

Quinidine, cincophen, and quinine may exert a direct hypoprothrombinemic effect that potentiates bleeding in anticoagulated patients. Meprobamate, monoamine oxidase inhibitors, neomycin, and a variety of other agents have been reported to alter effects of anticoagulants but controlled studies in man are lacking. Salicylates both affect prothrombin synthesis and impair platelet function and should not be used in conjunction with coumarin anticoagulants. If a patient is chronically anticoagulated and requires a drug that will potentiate the coumarin effect, smaller than usual doses of the anticoagulant, the second drug, or both, should be given. If the patient is chronically taking both drugs, and the second drug is discontinued, more coumarin may eventually be required for a satisfactory prothrombin response.

Because many of the coumarin interactions with other drugs involve competition for sites of protein binding or hepatic metabolism, initiation of coumarin anticoagulation in a patient may also affect the handling of the agents he is already taking. Bishydroxycoumarin raises plasma levels of diphenylhydantoin and tolbutamide presumably by inhibiting hepatic metabolism of these agents, prolongs the half-life of chlorpropamide by competing for renal excretion, and by an unknown mechanism elevates serum phenobarbital levels in patients receiving large doses of phenobarbital.

Drugs that have been investigated in patients also taking anticoagulants and that have little or no interaction and are thus safe to give in usual dosage are acetaminophen, the benzodiazepine tranquilizers (86, 87), chlordiazepoxide, diazepam, and probably nitrazepam (88) and the diuretic chlorothiazide. Although meprobamate tends to reduce prothrombin times in chronically anticoagulated patients, this difference is clinically insignificant (89).

The easiest way to anticoagulate a patient is to use only the anticoagulant. This is not always possible, but therapy should be limited to drugs that are clearly needed. Successful anticoagulation of the ambulatory patient requires a good doctor-patient relationship and a cooperative patient capable of following instructions. One physician must maintain primary responsibility for the patient and all medications prescribed by others must be cleared by him. A good general rule is that no other medications of any kind are to be taken ever—this includes over-the-counter preparations, food additives, vitamins, and remedies offered by well-meaning friends and relatives without the permission of this physician. This does not mean that treatment for other ailments be denied, but the physician must give an anticoagulated patient a few extra minutes periodically to answer questions and check on the medications. If the doctor is too busy or unable to give this attention, someone who is willing to devote the effort should care for the patient. If the patient is either unreliable or uncooperative, he is a poor risk and should not be anticoagulated.

If a patient clearly requires treatment with drugs known to interact, these may be given, but the dose should be adjusted in the anticipated direction. If the potential for interaction between the two drugs is unknown, therapy should be instituted with small doses under careful observation and modified as indicated.

### ANTICONVULSANT THERAPY

Measurement of serum levels of diphenylhydantoin (DPH) indicates that, on the average, a range of 10–20 $\mu$g/ml produces seizure control with the fewest side effects (90). Levels below this range tend to be associated with poor control of seizures, while higher serum levels tend to be associated with nystagmus, ataxia, and lethargy (91). Coma may be seen at concentrations exceeding 50 $\mu$g/ml. Elimination half-lives which range from 10 to 24 hr are dependent on the rates of hepatic hydroxylation, which vary in individuals (92). The capacity for parahydroxylation may be limited (93). Because of the long half-life, a single daily dose may provide seizure control as effective as that obtained by divided doses. Rarely, a patient may be genetically unable to parahydroxylate this drug at all, and neurotoxic levels of the unchanged parent drug accumulate. Extremely rapid inactivators have also been identified (91).

It is difficult to determine whether diphenylhydantoin in man commonly induces its own metabolism or that of drugs handled by the same system, since published data are conflicting. Kutt (91) states that DPH is not a potent inducer in man. Inducer drugs such as phenobarbital and carbamazepine (94) may result in accelerated DPH inactivation in some but not all patients. While DPH is expected to compete and interact with drugs sharing a common metabolic pathway, the interaction between DPH and isoniazid involves agents handled by two different subcellular systems. Very slow acetylators of isoniazid (acetylated in the soluble fraction of liver) manifest diphenylhydantoin toxicity with customary doses of both drugs (95). The mechanism for this interaction is unknown. Practically, a slow metabolizer of isoniazid may require lower than usual doses of DPH to attain a therapeutically effective blood level.

When the pharmacokinetic data are considered, the common practice of administering diphenylhydantoin and phenobarbital together to improve seizure control is rather remarkable. Addition of phenobarbital to patients receiving DPH tends to result in lower serum levels of DPH in many patients, presumably by induction. In some patients DPH levels remain unchanged, while in others it increases to high levels (91, 96, 97). Some children chronically receiving phenobarbital developed high phenobarbital levels when DPH was added. These effects are variable and it is impossible to predict the effect in a particular patient. The mechanisms responsible for these variations have not been determined. The only sensible conclusion to be derived from these data is that anticonvulsant therapy is best initiated with either phenobarbital or diphenylhydantoin alone. If the patient has achieved steady-state conditions with respect to the drug and therapy has not afforded control of seizures, blood level determinations are required to adjust doses which provide optimum serum drug levels. If seizures are still uncontrolled, the second drug may be added and the response evaluated.

DPH is bound extensively to plasma proteins (more than 90%) and binding does not vary among individuals (98). In uremic patients, compared to normal individuals, DPH binding is decreased (99) resulting in low serum concentrations of parent drug and elevated concentrations of the major hydroxylated metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH) (100, 101). This is related to the degree of azotemia but not to serum protein concentrations (99). The short plasma half-life of DPH in uremic subjects suggests that the metabolic disposition of the drug is altered. The binding of DPH to albumin from uremic patients is enhanced after isoelectric focusing, but not by dialysis or electrophoresis (17). There is not enough information at present to determine whether dose recommendations should be changed for uremic patients. The effect of DPH at the pharmacologic receptor site of uremic patients is unknown. Is accumulation of HPPH related to increased toxicity? In vitro studies of rat brain subcellular fractions suggest that DPH may inhibit microsomal ATPases, which may be related to toxic effects. High calcium concentrations inhibit this affinity. The effects of HPPH on such subcellular fractions are unknown and should be investigated.

While pharmacokinetic information promises more effective seizure control, enthusiasm must be tempered by attention to other effects of diphenylhydantoin (102, 103). In the absence of megaloblastic anemia, serum and red cell folate levels are decreased in many patients in chronic therapy. Administration of folic acid to such patients may alter the disposition of DPH (104). Under physiologic conditions in man, DPH interferes with intestinal absorption of folate (105). Elucidation of the exact mechanism is awaited. It has been suggested that in patients with megaloblastic anemia, intestinal folate conjugase is inhibited, thus interfering with conversion and subsequent absorption of dietary folate. Another explanation offered for reduced serum folate levels is enhanced hepatic metabolism by the process of enzyme induction (106). Following DPH treatment, the normal insulin response to a sustained glucose load is impaired (107). Patients at risk of developing diabetes or who are simultaneously receiving other diabetogenic agents with DPH should be studied to establish whether DPH therapy will increase the risk of serious carbohydrate intolerance. Diphenylhydantoin therapy interferes with the response to the low dose, but not the high dose dexamethasone test in the evaluation of Cushing's syndrome associated with adrenal hyperplasia (108), possibly by alteration of dexamethasone metabolism. The ability of DPH therapy to accelerate cortisol metabolism has been used beneficially to ameliorate the symptoms of excessive steroids in Cushing's syndrome. Approximately 30% of institutionalized children receiving diphenylhydantoin in combination with other anticonvulsant drugs displayed the symptom complex of coarse facies, calvarial thickening, and elevated alkaline phosphatase values, which correlate with high drug levels as well as prominent gingival hyperplasia (109). An association between elevated alkaline phosphatase levels and hypocalcemia was found in another study of a similar institutionalized population. The suggestion has been made, but not proved, that anticonvulsant therapy may alter vitamin D metabolism in the liver (110) (or in the gut), resulting in abnormalities in bone and calcium metabolism.

The most frightening potential side effect of long-term anticonvulsant therapy with diphenylhydantoin may be the development of true malignant lymphomas (103). While cases of pseudolymphomas have been considered hypersensitivity reactions to anticonvulsant therapy, long-term administration may predispose the patient to the development of true malignancies. Patients with malignant lymphoma were four times more likely to have received DPH (2.3%) than a control population (0.6%). Since DPH has been in use for only 30 years, it is possible that such long-term manifestations are just now becoming apparent. Impairment of cellular and humoral immunity, stimulation of cortisol metabolism, and altered folate metabolism (which could affect nucleotide and DNA synthesis) occur with chronic anticonvulsant therapy. Diphenylhydantoin is a useful and effective drug, and the incidence of lymphoma is low, if truly increased at all. Careful attention must be paid to the development of lymphadenopathy in patients under treatment. Unnecessary long-term therapy should be avoided, and the drug discontinued whenever the patient's clinical condition permits.

## DRUGS USED IN CARDIOVASCULAR DISEASE

Pharmacokinetic studies in patients with cardiovascular disease suggest that many therapeutic failures as well as toxic reactions of familiar drugs can be avoided. Information on individual differences in plasma levels and metabolism dependent on the route of administration is also being used to establish effective dosage regimens for optimal study of new agents. Some of the more important findings will be outlined here.

*Antiarrhythmic agents.—*

*Quinidine*: Plasma levels of 3–6 μg/ml are usually effective and concentrations exceeding 10 μg/ml tend to be highly toxic (111). Quinidine is highly bound to plasma albumin and therefore only 20% of the plasma concentration is available in free, pharmacologically active form. Plasma concentration measurements must be interpreted in this light.

Prompted by an observation of quinidine toxicity in a patient receiving ordinary doses, a study relating quinidine excretion to changes in urinary pH revealed markedly reduced excretion of quinidine in alkaline urine with pH greater than 7.5 (112). Although the blood pH did not change, serum quinidine levels and toxic manifestations increased. Especially when higher doses are used, urinary pH may be an important factor affecting the successful outcome of therapy.

*Procainamide*: The extension of antiarrhythmic therapy to include prophylaxis of arrhythmias following acute myocardial infarction has stimulated studies to insure safe use of procainamide. Absorption, distribution, and rates of elimination vary among individuals, but the magnitude of these differences is less than that observed with other types of drugs (see tricyclic antidepressants) (111). Both therapeutic and toxic effects correlate well with plasma concentrations, which are usually effective between 4–10 μg/ml, are occasionally toxic between 8–10 μg/ml, and are usually toxic above 16 μg/ml (111).

Because the biological half-life of procainamide averages 3.5 hr, the drug must be administered frequently, every 3 hr, to maintain optimal therapeutic plasma levels. Steady-state plasma concentrations will be approached after four doses, administered every 3 hr. A total daily dose of 50 mg/kg body weight will produce therapeutic concentrations in plasma for most patients.

Koch-Weser has made the interesting observation that different plasma levels are required to control different arrhythmias. Lower levels are required for control of atrial than for ventricular disturbances, and premature ventricular contractions are suppressed by levels lower than that required for control of ventricular tachycardias.

While procainamide is generally well absorbed within the first 24 hr following myocardial infarction and in very ill patients, absorption of oral doses may be erratic. Intramuscular doses are well absorbed and are preferable under these conditions. Severe decreases in cardiac output and arterial pressure result in delayed intramuscular absorption and here intravenous therapy is preferred, avoiding a rapid infusion. To reach therapeutic levels quickly, an intramuscular priming dose, twice the maintenance dose, is useful. In low cardiac output states and prerenal azotemia, the volume of distribution and the elimination rates of procainamide are decreased and lower doses must be used. A high percentage of procainamide is excreted unchanged by the kidneys; with impaired renal function plasma half-life and plasma levels are increased.

Plasma procainamide concentrations are technically simple to determine. The values obtained are helpful in establishing optimal doses for the individual patient and are especially valuable in regulating therapy for patients who have not responded to therapy or those with decreased cardiac output, renal disease, or rapidly changing clinical states.

The metabolism of procainamide in man is still under investigation. The drug does not appear to increase its own metabolism during chronic therapy. The major metabolite in man is N-acetylprocainamide, which constitutes about 50% of the excreted dose (113).

A lupus erythematosus-like syndrome (LE) has been associated with procainamide as well as other drugs. Antibody to denatured DNA and nucleohistone was found in sera from patients with procainamide-induced LE (114). It might be of interest to ascertain whether procainamide or a metabolite is bound to DNA obtained from such patients and whether or not this material has enhanced immunogenicity. Further work is needed to determine whether a positive correlation exists between serum or tissue levels of procainamide and its metabolites and the development of this syndrome.

*Lidocaine*: Lidocaine is effective therapeutically with blood concentrations ranging between 1.2 and 5.5 μg/ml, which for most patients can be achieved by a 100 mg intravenous bolus followed by an infusion rate of 20 to 50 μg/kg/min. A single IV dose of lidocaine, like many other drugs, disappears in two phases. During the first 7 to 20 min, which corresponds to the brief, clinically observed period of pharmacologic activity, the decline is rapid and probably reflects distribution to tissues as well as some metabolism (115). The elimination of the drug from the plasma by metabolism and excretion is 87–108 min and is much longer than would be expected from the drug's clinical effects.

This long elimination half-life may be important in establishing dose regimens for patients with severe disease. Steady-state arterial plasma concentrations of lidocaine are higher in patients with poor cardiac function as measured by cardiac index (116). In advanced heart failure, reduction of both the volume of distribution and plasma clearance of lidocaine are probably a reflection of decreased uptake by the liver. The liver accounts for removal of 70% of lidocaine from the blood, probably by metabolism, and a low cardiac index is associated with reduced hepatic blood flow. Clearance of lidocaine from the blood was decreased in patients with alcoholic cirrhotic liver disease, but the volume of distribution of the drug was normal (115).[4] Thus, in advanced congestive failure or other states with decreased or abnormal hepatic blood flow, the elimination time of lidocaine may be lengthened, and the plasma levels may be higher than otherwise expected from the apparent duration of pharmacologic activity. Blood level determinations and half-life calculations are helpful in planning optimal dosage schedules for such patients.

After oral administration of lidocaine, approximately 35% of a given dose reaches the systemic circulation, the remainder presumably being taken up by the liver during the first passage of drug (first pass effect) (117). Subjective symptoms are experienced by patients at plasma concentrations lower than those following intravenous therapy, which suggests metabolite formation during the first passage through the liver. Measurement of urinary metabolites after oral lidocaine in man indicate that 4-hydroxy-2,6-dimethylaniline accounts for 70% of the dose administered and probably results from hydrolysis of the amide bond of lidocaine or one of its dealkylated metabolites. Monoethylglycinexylidide, another metabolite of lidocaine, has been reported to have emetic and antiarrhythmic properties. This metabolite is likely to be a major intermediate in the metabolism of lidocaine in man, and if it is proven to have antiarrhythmic and toxic activity, an explanation may be provided for some of the effects of continuous lidocaine therapy. Because lidocaine is largely biotransformed by the liver, little is excreted unchanged by the kidneys and, as one might predict, there is no significant alteration of lidocaine kinetics in advanced renal failure.

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[4] I wonder to what extent the decreased plasma clearances reflect vascular shunting, effectively bypassing the liver, rather than impairment of the liver tissue itself to metabolize the lidocaine, in the particular type of cirrhotic patient selected for study. There is insufficient published data at this time to generalize that lidocaine handling is impaired in all patients with liver disease, but it does appear to be altered in advanced cirrhosis or other states such as severe congestion, where hepatic blood flow is abnormal.

Patients taking anticonvulsant medication or phenobarbital have lower plasma lidocaine levels at 30 and 60 min than control patients taking no premedication (118). If the metabolites of lidocaine are inactive these patients may be more resistant to the effects of usual doses. However, if the metabolites are active pharmacologically, the toxic or therapeutic effects may be exaggerated. The significance of this interaction remains to be discerned.

*Propranolol.*—During its popular use as an antihypertensive, antianginal, and antiarrhythmic agent, wide variations in propranolol doses have been required for achievement of a given degree of $\beta$ blockade (119). Recent studies designed to further understanding of the individual differences in dosage requirements have yielded some fascinating but complicated results. Following administration of a single oral dose of 30 mg or less of propranolol, only trace amounts of the drug are detected in the systemic circulation (120), possibly reflecting essentially complete hepatic extraction (first pass effect). With a larger single oral dose or with chronic dosing, a smaller fraction (72%) will be extracted during the first pass and thus proportionately more of the drug will appear in the systemic circulation. Shand predicts that the drug should thus accumulate during chronic therapy and supports this with data indicating three- to fourfold increases in plasma levels after repeated doses (121).

Although existing assays for propranolol and some of its metabolites have provided useful information, clarification of differences in propranolol metabolism requires assays with greater sensitivity. Existing methods have detected 4-hydroxypropranolol and napthoxylactic acid as well as a number of unidentified metabolites. Gas chromatography-mass spectrometry analysis has identified more than 17 metabolites of propranolol in man, which are formed by hepatic and extrahepatic microsomal systems, as well as mitochondrial and serum enzymes (122). Two hours after a single oral dose of propranolol, 4-OH propranolol, which has $\beta$-blocking activity, is found in the blood, and the levels of parent drug are less than those required for a given degree of $\beta$ blockade, which suggests that this active metabolite is effective in vivo. The importance of this metabolite during repeated dosage is not clear from existing studies. Two populations may exist with respect to dose levels required to achieve a given degree of $\beta$ blockade on chronic, oral propranolol therapy. One group of patients requires high plasma levels of propranolol for a given degree of β blockade, while the other group of patients requires relatively low plasma concentrations (123). Whether these differences reflect genetically determined differences in propranolol metabolism, i.e. the low plasma concentration group synthesizing an active metabolite with β-blocking activity, or possibly deconjugating inactive metabolites and converting them to active metabolites, remains to be elucidated.

During short-term intravenous therapy, 4-OH propranolol does not appear to be synthesized (120). To effect the same degree of β blockade during single dose intravenous propranolol therapy, blood levels three times higher than that required during single dose oral therapy are needed. The plasma half-life was found to be 2.3 hr after intravenous therapy but 3.2 hr after oral therapy. Continuing absorption was offered as an explanation of the longer half-life with oral therapy.

Because of the wide variation, blood level determinations of propranolol are not helpful unless the plasma concentration can be related to the degree of β-adrenergic blockage by one of several relatively simple tests available.

Although a recent study of propranolol given to patients in renal failure suggested no therapeutic modifications, the published half-lives were very prolonged in occasional patients (124). Information on whether elimination half-lives of propranolol are altered with chronic therapy and studies of individual variation in extrahepatic propranolol metabolism are needed. Phenothiazines and tricyclic antidepressant pretreatment of rats inhibited hepatic microsomal metabolism of propranolol, probably due to competition for the same sites of metabolism.

*Cardiac glycosides.*—Withering described the cardiac glycosides 200 years ago, but only in the last decade have assay techniques developed sufficiently to permit investigation of the clinical pharmacology of these drugs. This discussion will be limited to the two most widely used agents: digoxin and digitoxin.

Digoxin differs from digitoxin only by the presence of a hydroxyl group in the $C^{12}$ portion of the steroid nucleus, rendering it more polar. This property, however, is probably responsible for important differences in metabolism and excretion. Both compounds are nearly completely absorbed from the gastrointestinal tract, and the therapeutic effect is not affected by the presence of food in the stomach. Malabsorption due to hypermotility or mucosal defects, but not pancreatic disease, will result in decreased plasma levels of digoxin (125).

Differences in bioavailability of tablets of digoxin from commercial sources resulted in serum levels differing by a factor of seven when tested in the same individuals (126). Since toxic and therapeutic levels vary only two- to threefold, such differences serve to further complicate pharmacokinetic studies and a successful outcome of therapy.

Digoxin is not bound to serum proteins to any extent, while digitoxin is highly bound (90%). The elimination half-life (metabolism and excretion) of digoxin is 1.6 days and, in contrast to lidocaine, closely approximates the physiologic half-time (127). Digoxin does not appear to be extensively metabolized or undergo any appreciable enterohepatic circulation. Thirty-five percent of a given dose will be lost 24 hr later, largely by urinary excretion of unchanged digoxin.

The elimination half-life of digitoxin is much longer than that of digoxin, tends to vary in individuals, and averages between 4–6 days, which corresponds to the physiologic half-life and the chloroform-soluble, cardioactive metabolites (128). Interindividual differences are likely to be better understood as the complexities of digitoxin metabolism are unravelled. Daily, more than 60% of digitoxin is biotransformed to inactive metabolites, 8% is transformed in the liver to an active metabolite, digoxin (12-OH digitoxin), and 30% is excreted unchanged in the urine. A significant enterohepatic circulation of digitoxin in man has not been considered in many of these metabolic studies (129).

Phenobarbital pretreatment in man was found to decrease the steady-state plasma level of digitoxin by 50%, shorten the half-life, and increase the conversion of digitoxin to digoxin (130). Clinically encountered concentrations of phenylbutazone, warfarin, tolbutamide, and diphenylhydantoin were associated with reduced digitoxin serum concentrations. Elucidation of how these drugs affect the cardioactive metabolites of digitoxin is needed for proper clinical perspective. Clinically encountered concentrations of phenylbutazone, warfarin, tolbutamide, sulfadimethoxine, and chlorophenoxyisobutyric acid have no effect on displacing digitoxin-$^3$H from human albumin in vitro.

Because digoxin is largely dependent on renal excretion for elimination of the active drug from the body, impairment of renal function results in a longer half-time and a slower disappearance from the body. In a totally anuric patient, the half-time will be prolonged to 4.4 days and only 14% of a given dose will be eliminated in 24 hr by nonurinary routes. Jelliffe, in a now classic paper, has shown that digoxin elimination is proportional to creatinine clearance and/or blood urea nitrogen (BUN) and has devised a simple mathematical model for maintaining constant body stores of digoxin when renal function is impaired or rapidly changing (131). By reading simple graphs or making simple calculations, the method is designed to maintain a therapeutic effect while minimizing toxicity. In a recent study of elderly, digitalized, ambulatory patients, however, a high degree of impairment of renal function was found but plasma digoxin levels were unrelated to BUN or creatinine clearance (132). In this study, digoxin half-times were not measured. Digoxin clearance was less than creatinine clearance in these elderly subjects. Interestingly, despite the impairment of renal function found, only two patients (who were asymptomatic) required reduction in dose as a result of plasma level measurements.[5] Diuretic therapy did not appear to alter digoxin handling in any systematic way. In a third study, the same dose of digoxin was given to old and young subjects, and the handling was compared. Digoxin and creatinine clearances were similar in elderly subjects, but in young, healthy subjects the creatinine clearance exceeded the digoxin clearance (133). After 24 hr of therapy, the elderly subjects manifested prolonged blood half-lives and had higher plasma levels. It would not be surprising if more than one human genetic population exists with respect to digoxin handling, particularly with respect to tubular reabsorption.

[5] In these two studies, plasma digoxin is measured by different methods.

Because of appreciable elimination by biotransformation, digitoxin elimination is less affected by renal disease than is digoxin. As with digoxin, Jelliffe has correlated a reduction in creatinine clearance with a slower digitoxin disappearance from the body, prolongation of the apparent half-life of elimination, and loss of a smaller percentage from the body each day (134). An anuric patient with normal liver function loses only 7.6% of digitoxin stores each day, which corresponds to a half-life of 9 days. While the conversion of digitoxin to digoxin has been considered in the calculations, the role of the enterohepatic circulation has not. Digitoxin metabolism will be altered by liver disease, and Jelliffe's method is recommended only for patients with normal liver function. In contrast to this work, the elimination of digitoxin was not significantly different in normal individuals and patients with impaired renal function in a study by Rasmussen and co-workers (135). Serum digitoxin levels were significantly lower in patients with impaired renal function, and most of the differences were ascribed to lower serum proteins and thus altered digitoxin binding. Tritiated digitoxin bound to plasma from uremic patients by 86% while plasma from healthy volunteers bound 90% (17). Shoeman & Azarnoff used these numbers to calculate the concentration of unbound digitoxin in the uremic patients cited in Rasmussen's study, and found the concentration of unbound digitoxin in the uremic individuals to be equal to or higher than in patients with normal renal function. Nondialyzable inhibitors may be present in uremic plasma and may be removed from albumin by isoelectric focusing. Further work is required to evaluate this.

The practice of giving larger doses of digoxin to hyperthyroid patients has been supported by serum level determinations. Hyperthyroid patients had higher serum levels of digoxin when compared to euthyroid individuals given the same dose, probably due to differences in distribution.

The application of the radioimmunoassay for digitalis glycosides has served to stimulate studies of the prevalence, epidemiology, and treatment of digitalis toxicity. Interpretation of digoxin levels obtained by radioimmunoassay suggested that most patients without clinical evidence of digoxin intoxication will have serum levels of 2.0 ng/ml or less, while most patients with clinical toxicity had levels higher than 2.0 ng/ml. (136). Most patients without digitoxin toxicity had levels below 26 ng/ml (128). However, these assays are not helpful unless viewed in the entire clinical perspective. In two recent prospective studies, blood level criteria alone could not be used to distinguish toxic from nontoxic individuals (137, 138). Forty-one percent of hospitalized patients with definite digitalis toxicity died. The toxic patients had more severe cardiac or pulmonary disease than nontoxic patients, and a lower serum albumin, but no significant differences between toxic and nontoxic patients were found regarding type of digitalis preparation, diuretic therapy, serum potassium, or multidrug regimens. Serum creatinine levels were higher in toxic individuals who had received digoxin, but not in patients receiving digitoxin or digitalis leaf. Thirty percent of patients clinically classified as definitely toxic had a serum digoxin level of less than 1.7 ng/ml or a digitoxin level of less than 25 ng/ml. Fifteen percent of patients not considered toxic had high serum levels. This study emphasizes the possibility of interindividual differences in drug handling due to the underlying disease state and correctly cautions that diagnosis of digitalis toxicity is still best made by clinical criteria. Reliance on blood level determinations out of clinical context is dangerous. The usefulness of these assays is likely to increase as all the complexities of glycoside handling, metabolism, and interactions become clearer. Clinically, it is appreciated that different doses of digitalis are required to treat heart failure (inotropic effect) and slow the ventricular rate in atrial fibrillation (AV conduction). Future studies are likely to indicate different therapeutic and toxic levels that will be related to the underlying disease. This is somewhat supported by the finding that patients with AV block as a manifestation of digoxin toxicity had lower serum levels than patients who manifested toxicity by ectopic impulse formation (136). Patients with clinical digoxin toxicity tended to have coronary artery disease and were receiving antiarrhythmic drugs such as quinidine and propranolol. Much work is needed in the study of the effects of other commonly used drugs in digitalized patients.

Recent administration of radioisotopes is a source of potential error in the radioimmunoassay. This can be checked by additional isotopic counting setting.

A second test that may be helpful in confirming a clinical impression of digitalis toxicity is the measurement of the product of salivary potassium and calcium (139). It would be interesting to see how the salivary electrolytes compare with blood glycoside levels as determined by radioimmunoassay, and whether glycosides are present in saliva.

One of the most exciting areas in clinical pharmacology is the theoretical possibility of reversing drug toxicity in man by the application of immunologic and other techniques. Death was prevented and clinical arrhythmias reversed in dogs given lethal doses of digoxin and then treated with digoxin-specific antibodies (140). At near stoichiometric concentration of digoxin, purified antibody or its Fab-Fe fragment was capable of reversing ventricular tachycardia in intact dogs (141).

While a few patients are known to have antibodies in vivo, along with increased drug requirements, the extent or importance of this as a clinical problem is not currently known.

Spironolactone and other steroids can prevent digitalis toxicity in experimental animals, and spironolactone pretreatment of rats results in a reduction of tissue and serum levels of tritiated digitoxin (142). The mechanism of action is not yet established.

Cholestyramine (143a, 143b) and colestipol (144), binding resins, may be of use in treating digitalis toxicity. Cholestyramine treatment effects a reduction of the half-life of digitoxin and its cardioactive metabolites, which correlates with changes in left ventricular ejection time and duration of electromechanical systole. This alteration is believed to be mediated by interruption of the enterohepatic circulation. Since digoxin does not undergo an appreciable enterohepatic circulation, the success of colestipol in altering digoxin excretion suggests that the mechanism of action is not yet fully understood.

## ANTIHYPERTENSIVE THERAPY

Treatment with combined vasodilatation (hydralazine) and β-adrenergic blockade (propranolol) is effective in refractory severe essential hypertension in ambulatory patients (145). In patients treated with hydralazine alone, the plasma concentration, but not the dose administered, correlated well with the hypotensive effect (146). The relation between dose and plasma level of hydralazine varies fourfold between individuals but remains constant in the individual patient receiving chronic therapy. Hydralazine is acetylated by hepatic N-acetyltransferase, the same system which handles isoniazid and sulfamethazine. Like isoniazid, the population is divided into slow and fast acetylators of hydralazine. Slow acetylators had higher blood levels than fast acetylators, and fast acetylators required doses twice as high for satisfactory blood pressure reduction. Patients with impaired renal function tended to have high blood levels of hydralazine. The lupus-like syndrome is much more common in slow acetylators of hydralazine. and high blood levels may be important in the development of this syndrome (147).

## PSYCHOTROPIC DRUGS

One of the most challenging problems of drug therapy is the satisfactory treatment of the psychotic patient. The ideal drug would achieve a rapid therapeutic effect with a minimum of side effects and restore the individual to a functioning capacity. While great strides have been made in the development of psychopharmacologic agents, these drugs have not been utilized to their full advantage. Recent work promises that optimum use of our currently available psychotherapeutic agents will be forthcoming in the foreseeable future.

*The phenothiazines.*—While the phenothiazines are generally considered to be effective, little is known to explain why some patients respond to this class of drugs while others do not. Lack of a sensitive assay for measuring chlorpromazine and its metabolites has been an obstacle to the study of this problem. Recently, Chan & Gershon have developed a direct thin layer chromatographic scan technique which permits the quantitative and qualitative analysis of chlorpromazine and seventeen of its metabolites in body fluids (148a, 148b).

The preliminary findings of these workers promise to distinguish responders from nonresponders (149). Nonresponders appear capable of converting more of the parent compound, chlorpromazine, to its inactive sulfoxide form. Large amounts of this sulfoxide, despite adequate levels of the unmetabolized parent drug, may interfere with achieving the desired effect. Moreover, the source of this metabolite may be a tissue other than the liver. As these workers extend their observations, they approach the exciting possiblity that an in vitro blood test will predict in advance whether a patient is likely to respond to chlorpromazine.

The phenothiazines are inducers of hepatic microsomal drug metabolism. To decide whether drug metabolism could be further enhanced by treatment with other inducers, 180–300 mg/day doses of phenobarbital were administered for two to seven days to hospitalized psychiatric patients being chronically treated with chlorpromazine. Excretion of predominantly conjugated metabolites increased by 10 to 81% (150). Patients who normally received maintenance barbiturates in addition to chlorpromazine excreted less chlorpromazine when the barbiturates were withdrawn for seven days. Magnesium- and aluminum-containing antacids presumably decrease chlorpromazine absorption and should not be administered at the same time (150).

*The tricyclic antidepressants.*—The availability, within the past decade, of a sensitive analytical method has enabled Sjoqvist and his co-workers to study the clinically observed marked individual differences in the response to tricyclic antidepressants. Patients treated with the same dose of drugs such as desmethylimipramine or nortriptyline sometimes differed as much as thirtyfold in their apparent steady-state plasma levels. In contrast to the phenothiazines, insignificant amounts of the parent drugs are excreted in urine and individuals do not differ with respect to absorption. Measurement of steady-state plasma levels of nortriptyline in identical and fraternal twins suggested that the metabolism of this drug is genetically controlled. Using gas chromatography and mass spectrometry, Hammar and co-workers have identified nortriptyline and its metabolites in body fluids from treated patients. The excretion rates of the main metabolite of nortriptyline, 10-hydroxynortriptyline, were parallel to the overall plasma elimination rate of the parent drug (151). Treatment with various drugs such as barbiturates lowered plasma concentrations of nortriptyline, presumably by inducing further drug metabolism.

A number of important findings which deserve immediate clinical application have resulted from these studies. Despite the large variation in plasma levels between individual patients, in any given patient the plasma level tended to be constant in the absence of therapy with other drugs. There was a significant positive correlation between the plasma level of nortriptyline and the subjective side effects experienced by the patients (152). There was no correlation between the dose prescribed and side effects. Optimal amelioration of depression correlated with plasma levels ranging from 50 to 139 ng/ml. Plasma levels lower or higher than this range had no effect on depression. Very high plasma levels appeared to potentiate the depression for which the patient was being treated in the first place (153). One cannot help but wonder as to the number of patients who empirically are given more tricyclic antidepressants when they really require less!

Steady-state plasma concentrations of nortriptyline and desmethylimipramine can be accurately predicted in all subjects studied from single-dose plasma level data if the rate of elimination and apparent volume of distribution are known (154). Alexanderson has studied the disposition half-life of single and multiple oral doses of nortriptyline and desmethylimipramine in the same subject. Despite differences in the apparent volume of distribution between these two drugs, presumably due to differences in protein binding, the ratio between the steady-state plasma concentration of desmethylimipramine and nortriptyline is constant in all subjects. In the absence of major changes in environmental factors and the disease state of the patient, these values remain comparable over a period of many years

This is extremely useful in providing optimal therapy for a patient with depression, a disease which tends to recur with time.

Amitriptyline, a commonly prescribed tricyclic antidepressant, is metabolized by N-demethylation to pharmacologically active nortriptyline. Plasma concentrations of amitriptyline and nortriptyline measured in six patients treated for 6 weeks correlated well with clinical improvement (155). Individuals differed widely in plasma levels of both amitriptyline and nortriptyline. Patients who had plasma concentrations below 120 ng/ml showed a poor clinical response. In contrast to the study by Asberg (153), no upper limit of an effective plasma concentration was found.

Administration of tricyclic antidepressants may interfere with the handling of other drugs simultaneously received. Using normal medical student volunteers, Vesell and co-workers have shown that administration of nortriptyline results in a marked prolongation in the plasma half-lives of antipyrine and bishydroxycoumarin (156). Animal studies suggest that this is effected by a reduction in the rate of drug biotransformation in liver microsomes. When tricyclic antidepressants are administered together with other drugs handled by the hepatic microsomal system, the patient should be closely monitored. Reduction of the dose of one or both drugs may be required. Quantitation of optimal dosage by blood level determinations would be the ideal situation, but it is not always practical. It should be emphasized that there are large individual variations in the extent to which the normal volunteers prolong their antipyrine and bishydroxycoumarin half-lives and that it is difficult to predict in advance how a given individual will respond. Where blood level determinations are not available, it would seem prudent to initiate therapy with lower than usual doses and adjust optimal therapy according to the patient's response.

*Amphetamines.*—The metabolism of the amphetamines in human subjects is profoundly influenced by alterations of urine pH (157). The half-life of amphetamine was 9 hr during the production of acid urine, but ranged from 16 to 31 hr when the urine was alkaline and resulted in differences in metabolism. During acid urine production there was little metabolism and most of the drug was excreted within the first 24 hr largely unchanged. During alkaline urine production the longer persistence in the blood led to appreciable metabolism. The dependence of plasma amphetamine half-life on urinary pH may have an important clinical application in the treatment of amphetamine overdosage where acidification of the urine might be helpful in enhancing excretion.

*Monoamine oxidase inhibitors.*—The monoamine oxidase inhibitor, phenelzine, unlike tricyclic antidepressant metabolism in endoplasmic reticulum (microsomal fraction) of liver, is a substrate for liver acetyltransferase in the cytoplasm. Depressed patients were treated with phenelzine and their responses correlated with acetylator phenotypes. There was no relation between phenotype status and clinical improvement, but adverse effects were more common among slow acetylators (158).

*Interactions at the adrenergic neuron.*—This type of interaction illustrates a unique category of adverse drug effects and has created a new spectrum of cardiovascular disease. It is important to understand and remember this category, as the end result is often catastrophic morbidity or mortality.

Norepinephrine, the adrenergic transmitter, is synthesized within the nerve terminal and stored there in vesicles to prevent inactivation by the enzyme monoamine oxidase (MAO). In response to a stimulus, norepinephrine is liberated from the vesicles, passes out of the nerve terminal, and reacts with the alpha receptor. The action of norepinephrine at the receptor is terminated, not enzymatically, but uniquely by a pump mechanism that returns norepinephrine to the neuron where it is again stored until release by the next stimulus. This specialized recapture, which terminates norepinephrine action, is called the reuptake mechanism.

Amines such as tyramine, amphetamine, ephedrine, and metaraminol (Aramine) compete with norepinephrine for reuptake and also release intraneuronal stores. The result of this competition is a pressor effect, due to the prolonged action of norepinephrine at the receptor site.

The tricyclic antidepressants block norepinephrine uptake, but do not appear to affect norepinephrine stores. The antihypertensive, guanethidine, alters transmitter (norepinephrine) release in sympathetic postganglionic fibers that innervate arterioles, and thus lowers blood pressure. To exert its action, guanethidine enters the neuron by the same pathway responsible for the reuptake of norepinephrine and becomes concentrated in the storage granules. The indirectly acting amines and the tricyclic antidepressants inhibit the uptake of guanethidine into the neuron.

MAO is present in the gut and liver as well as in adrenergic neurons where it inactivates endogenous and exogenous amines. The MAO inhibitor drugs impair the activity of this enzyme. Tyramine, a natural amine present in many foods, is normally metabolized by intestinal MAO and therefore not significantly absorbed. Any tyramine absorbed is inactivated by hepatic MAO. In the presence of a MAO inhibitor, tyramine is absorbed by the gut, is not metabolized by the liver, and thus reaches the adrenergic neuron in large quantities. Intraneuronal norepinephrine is already increased due to inhibition of intraneuronal MAO. Tyramine enters the neuron via the reuptake pathway keeping more norepinephrine at the receptor site, and once inside the neuron, displaces increased quantities of norepinephrine resulting in a dramatic pressor effect.

*Antagonism of effect of guanidinium antihypertensives:* The simultaneous administration of tricyclic antidepressant drugs (159a) and the guanidinium antihypertensive agents, guanethidine and bethanidine, result in a loss of the antihypertensive effect. Patients receiving desipramine, protriptyline, imipramine, or amitriptyline will lose the antihypertensive effect of guanethidine, often in an insidious manner. This places the severely hypertensive individual at great risk. Less than one day's dose of desipramine will abolish the antihypertensive effect of guanethidine for the next week, and, as Oates points out, is a striking example of clinical drug antagonism.

Not only do the tricyclic antidepressants inhibit transport of guanethidine to its site of action, but many amines also antagonize the actions of guanethidine by preventing its uptake into the neuron and also by releasing intraneuronal stores of the antihypertensive. As many nonprescription decongestant, anti-asthmatic, anti-obesity, and cough preparations contain sympathomimetic amines and are often taken by patients in large quantities, patients under treatment with guanethidine and related drugs must be cautioned against taking any drugs without medical permission.

The tricyclic antidepressants, phenothiazines, and amines such as metaraminol inhibit the metabolism of guanethidine, debrisoquin, and guanoxan by a hepatic microsomal system in the rat. Despite this, the predominant clinical effect seen when these drugs are used concomitantly is inhibition of the transport of guanethidine to its site of action in the nerve terminal. Desipramine blocks the active uptake of guanethidine into adrenergic neurons and thereby prevents guanethidine's pharmacologic effect even though levels of guanethidine in the body of the rat are increased because of inhibition of the hepatic metabolism of guanethidine—resulting in a disparity between drug levels and pharmacologic effect (159).

*Interactions producing hypertensive crises:* Although MAO inhibitors cause hypotension when given alone, because they impair the ability to inactivate sympathetic and indirectly acting amines, dramatic and catastrophic pressor responses have occurred in patients ingesting such amines in foods containing high quantities of tyramine. The combination of MAO inhibitors and tricyclic antidepressants has been reported to be particularly hazardous, since both drugs act to greatly potentiate the pressor effect of norepinephrine and may occur even when the drugs are administered days apart. Psychiatric patients being treated with MAO inhibitors or tricyclic antidepressants must also be cautioned against amphetamine abuse. Widespread abuse of amphetamines in young people may provide a chilling source for hypertensive crises and cerebrovascular accidents if drugs acting on the adrenergic neuron are simultaneously abused.

The antibacterial agent furazolidone (Furoxone) is recommended for treatment of gastroenteritis but has been found to act as a MAO inhibitor. Although it has no MAO inhibiting effect in vitro, a metabolite of this drug is formed in vivo and is implicated in the enzyme inhibition. Thus, although the parent drug is not an MAO inhibitor, the patient is at risk of suffering possible MAO interactions.

*Treatment of the depressed cardiac patient:* The number of patients suffering from both heart disease and mental depression appears to be increasing, but the safety of psychotropic drug therapy in the cardiac patient has been questioned (160). Both the phenothiazines and tricyclic antidepressants, which are structurally similar, have been associated with electrocardiographic abnormalities, myocardial infarction, and sudden death. In a prospective study, a disproportionate number of deaths in cardiac patients being treated with amitriptyline has been reported (161). It is not stated whether the mechanism for these deaths might lie in a possible drug interaction. No evidence of an association between amitriptyline and adverse cardiovascular effects in patients with cardiovascular disease was found by the Boston Collaborative Drug Survey Program (162). The reasons for the disparate findings between these two groups is not immediately clear. It would be of interest to have steady-state plasma levels, knowledge of other drugs received, and information regarding possible impurities or differences in manufacturing practices.

Anatomic changes in the arterioles and arteriolar capillary beds in the hearts of patients on long-term phenothiazine therapy have also been described. While much information is still required, it would be prudent to avoid using phenothiazines and tricyclic antidepressants in cardiac patients when other therapy such as barbiturates, benzodiazepines, patience, and a kind word are effective. When phenothiazine and tricyclic therapy must be used, these agents should be used in the smallest effective doses and with careful attention to the cardiovascular status of the patient.

*Useful interactions of adrenergic drugs:* Methylphenidate, a mild antidepressant when given alone, has also been reported to inhibit hepatic drug metabolizing enzymes in human subjects. Wharton and co-workers used this agent in conjunction with tricyclic antidepressants to treat patients with recurrent refractory psychotic depressive illness. Patients were allowed to achieve steady-state blood levels of either imipramine or desmethylimipramine and then methylphenidate hydrochloride (Ritalin) was added to the regimen (163). Five of the seven patients treated with this combined therapy had striking clinical remissions, which was associated with a rise in the blood levels of the antidepressant drugs. In vitro studies suggested that for imipramine, methylphenidate inhibited hydroxylation to a greater extent than monodemethylation. Methylphenidate has also been used in conjunction with phenothiazine treatment to counteract sedative side effects. It is not known whether this action is due to a central or peripheral effect of methylphenidate.

Another area in which the possible useful interaction of adrenergic-acting drugs is being explored is the use of dextroamphetamine to predict response to the tricyclic antidepressants (164). Dextroamphetamine has a rapid but brief effect on mood. The tricyclic antidepressants require several weeks for a manifest effect on depression. A test dose of dextroamphetamine may have some predictive value in determining whether or not a patient is likely to respond to a course of tricyclic antidepressant drugs.

Schuckit and co-workers take a controversial stand and suggest that the combination of tricyclic antidepressant and MAO inhibitor may be effective in the treatment of refractory depression (165). The authors have extensively reviewed the literature as well their own cases and feel that the concern over adverse reactions involving the combination of tricyclic and MAO inhibitor therapy is not soundly supported by data. Further controlled studies in animals and man are advocated to substantiate whether this regimen, under controlled conditions, is effective therapy for psychotic patients refractory to other therapy.[6]

Perphenazine, haloperidol, and chlorpromazine decreased the urinary excretion of $^{14}$C-imipramine measured in eight patients. Perphenazine decreased the total urinary excretion of $^{14}$C-nortriptyline and its metabolites, decreased the plasma level of metabolites, and resulted in an increased plasma level of unchanged nortriptyline. Interestingly, it is not known whether this pharmacokinetic interaction has any clinical significance (166).

## DRUG INTERACTIONS IN PEDIATRICS

Special considerations for treatment of the pediatric patient, particularly the neonate, have been well publicized, especially competition between drugs and serum bilirubin. Recent studies of older patients with unconjugated congenital hyperbilirubinemia indicate at least two genetic populations with this disorder (167). Inducing drugs such as phenobarbital are being used therapeutically to lower the levels of serum bilirubin in such patients. Phenobarbital increases levels of hepatic microsomal uridine diphosphate glucuronyltransferase (168) and can also induce Y, a hepatic cytoplasmic organic anion-binding protein (which transports some drugs and bilirubin) in certain situations (30). Although the glucuronyltransferases involved in the conjugation of bilirubin and glucuronidation of drugs have been considered to be different enzymes, patients who respond to phenobarbital treatment may be of more than one genetic background since glucuronidation of drugs may be abnormal in the type II patients described by Arias, but glucuronide conjugation of salicylate, salicylamide, and acetaminophen as well as glycine conjugation or/and sulfate formation were normal in two patients recently reported by Levy (169).

Thaler and co-workers recently studied the effects of phenobarbital administration on the induction of NADPH cytochrome *c* reductase, which is a component of the microsomal electron transport chain involved in many drug oxidations (170). The amounts of this enzyme increased in normal subjects and in some subjects with liver disease, but not in three patients with chronic liver disease. Recently these workers found phenobarbital to be of value in treating the symptoms of idiopathic cholestasis (171) by a mechanism which remains to be determined.

Much concern has resulted from the findings that 3% hexachlorophene may be absorbed through intact skin of babies and results in central nervous system toxicity when administered to animals (172) or infants in excessive doses or where the skin has been denuded. Infants extensively washed with this chlorinated phenol derivative manifested serum concentrations that approached toxic levels obtained in the animal experiments. Until more is learned about the effects of this antibacterial agent, current recommendations in the United States confine the use of this agent to surgical and hand scrubs of medical personnel, and advise thorough rinsing and application to limited areas if specifically indicated in pediatric patients.

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[6] This exemplifies the type of controversy that has been engendered by the numerous reports of drug interactions in recent years. Several reports of serious side effects often stimulate countless other claims that these agents have been in use for years and are safe and effective. Such disputes can only be answered adequately by carefully controlled studies in which the drug or drugs in question are administered in a manner suitable for use under ordinary clinical conditions.

Possibly the most tragic drug interaction encountered recently is the development of adenocarcinoma of the vagina in adolescent girls whose mothers received the nonsteroidal estrogen, diethylstilbestrol, during the first trimester of pregnancy (173, 174). The afflicted patients are either dead or have been treated by pelvic exenteration. Possibly as further offspring of treated women reach maturity, additional cancers may be encountered.

## CONCLUDING REMARKS

Space limitations have precluded discussion of interactions with agents such as anesthetics, antibiotics, folate antagonists, hypoglycemics, alcohol, antituberculous drugs, sedatives, and drugs of abuse. These subjects, as well as the effect of drugs on the interpretation of laboratory tests, will be presented elsewhere (175). The excellence of recent efforts is reflected in the more effective use of antiarrhythmic agents, tricyclic antidepressants, and salicylate analgesics. A better understanding of phenothiazine, anticoagulant, anticonvulsant, and cardiac glycoside drugs appears imminent. Reidenberg's book on drug action and renal function is a lucid guide to the treatment of patients with altered renal function (176). Modifications of creatinine clearance determinations provide a rapid measure of this value (177) or a reasonable estimate when renal function is changing (178). That much remains to be learned is exemplified not only by the transplacental induction of carcinogenesis, but also by the finding that even phenobarbital, when given to asthmatics who require steroids, nullifies the therapeutic effect by enhancing steroid degradation (179).

Progress in therapeutics has been remarkable, but its attendant pitfalls awaken the clinician to some practical aspects of pharmacology and require the pharmacologist to be mindful of the effect drugs will have in ordinary and exceptional situations. The development of clinical pharmacology as a subspecialty underscores the need for learning more about individual differences in drug response and the action of drugs in different diseases. Greater interactions between clinicians, clinical pharmacologists, and pharmacologists can accomplish the goals of therapeutics.

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# AUTHOR INDEX

A

Abbo, G., 165
Abbott, J. D., 76
Abbott, W., 281-83
Abboud, R., 242
Abdulla, U., 171
ABELMANN, W. H., 145-52; 145, 146, 150, 405
Abelson, R. B., 94
Abodeely, D. A., 368, 374-76
Abrahamsen, A. F., 193
Abramowicz, T., 93
Abrams, B., 316, 317
Abrams, W. B., 404
Abuelo, J. G., 70
Acheson, J., 9
Ackerman, B. O., 191
Ackerman, S. J., 130, 144
Ackermann, E., 32, 33
Adam, P. A. J., 316
Adams, J. H., 11
Adams, P. H., 368, 374, 375
Adams, R. H., 310, 313
Adamson, R. H., 30, 36
Adler, W. M., 177, 193
Adlersberg, D., 316
Admirand, W. H., 413
Aggeler, P. M., 393, 394
Agosto, G. M., 295, 297
Agostoni, E., 115
Aguilar-Parada, E., 303-6
Ahlberg, C. D., 392
Aicher, R. K., 129
Aisenberg, A. C., 329
Akiyama, Y., 165
Aksoy, M., 79
al Awqati, Q., 20, 21, 104
Albert, A., 295
Albertini, R. J., 52, 140
Albright, F., 224
Alcala, R., 124
Aldrich, R. A., 191
Aledort, L. M., 1, 2
Alessandri, M., 10
Alexander, D., 90
Alexander, D. P., 229
Alexander, J. C., 379
Alexanderson, B., 408
Alfrey, A. C., 228
Alkjaersig, N., 335-39
Allan, W. S. A., 78
Allard, C., 9
Allderdice, P. W., 68
Allen, J. C., 379, 381
Allen, J. L., 294
Allen, R. J., 13
Allibone, E. C., 191
Almgård, L. E., 202
Alpert, S., 389, 413
Altemeier, W. A., 177, 193
Ammann, A. J., 188
Ammann, P., 188
Amos, D. B., 136, 138, 193
Amos, H. E., 179
Amsel, L. P., 391
Anastasi, A., 98
Anders, J. M., 63
Andersen, E., 77
Andersen, V., 137
Anderson, A. E. Jr., 121, 127
Anderson, D., 317
Anderson, F., 328
Anderson, J., 58
Anderson, J. L., 140
Anderson, M. S., 261, 262, 358-61
Anderson, R. C., 64
Anderson, V., 202
Andreoli, V. M., 9
Andrew, E. D., 42
Andrews, J., 390
Angell, R., 91
Annell, A. L., 93
Ansel, R., 209, 212
Ansel, R. D., 211
Anthonisen, N. R., 125, 126
Antia, A. U., 64
Antoniades, H. N., 233
Aoki, N., 11
Arbit, J., 209, 212
Arcila, H., 368
Ardeman, S., 399
Ardlie, N. G., 8
Arias, I. M., 36, 37, 388, 389, 413
Arias, L., 410
Armitage, P., 210
Armstrong, K. J., 237
Armstrong, W. D., 196
Arnaud, C., 228
Arnaud, C. D., 219-24, 227
Arnold, K., 397
Arrighi, F. E., 70-72
Arroyave, G., 317
Arseneau, J. C., 331
Artenstein, M. S., 191
Asberg, M., 390, 408, 409
Ashley, C. J., 397
Ashmore, J., 233
Ashton, W. L., 8
Ashwell, C. G., 296
Assan, R., 306
Aster, R. H., 1
Astin, T. W., 128
Atac, A., 3
Athens, J. W., 196
Atkinson, H. R., 76
Auerswald, W., 316
August, C. S., 141, 190
Aukland, K., 366, 367
Aungst, C. W., 76, 286, 327
Aurbach, G. D., 217, 218, 220, 222-24
Austen, K. F., 186, 191
Avelar, E., 369-73
Averette, H. E., 345, 348-50
Averill, R. L., 356
Avery, E. H., 374
Avioli, L. V., 227
Axel, R., 265
Axelrod, J., 409
Axtell, L. M., 331
Ayoub, E. M., 177, 193
Azakizawa, M., 361
Azarnoff, D. H., 385
Azarnoff, D. L., 387, 398, 405
Azurin, J. C., 105

B

Bach, F. H., 136, 140
Bachmann, R., 188
Badawl, H., 8
Baehner, R. L., 196
Baer, H., 195
Baetz, A. L., 106
Bagley, C. M , 332
Bagshaw, M. A., 327
Bahl, O. P., 295, 297
Baikie, A. G., 91
Bakay, B., 47, 49, 51, 52, 54, 55
Bakemeier, R. F., 328
Baker, D., 93
Baker, H., 347
Bakers, R., 261
Bakken, A. F., 245, 247
Balagura-Baruch, S., 372, 373
Baldini, M. G., 4
Balestieri, W., 320
Baliah, T., 36
Balis, M. E., 42, 46-48, 51, 52, 56
Ballester, D., 316, 317
Balsam, A., 317
Balten, J. C., 130
Bandura, A., 98
Bang, N. U., 5, 338
Bangerter, S., 360

Bansal, S. C., 202
Banwell, J. G., 19, 104-6
Barac-Nieto, M., 369-72
Barakat, M. J., 397
Baram, P., 176, 177
Barbeau, A., 210-12, 214, 259, 260
Barham, F. W., 234, 236
Barker, R., 76
BARLOW, G. H., 335-44; 336, 338
Barlow, J. J., 414
Barlow, M. H., 188, 191
Barlow, P., 68
Barnard, R., 106
Barnett, E. V., 193
Barnett, R., 168
Barnum, D. A., 106
Barr, A. N., 209, 210
Barr, D. P., 316
Barr, H. J., 68
Barr, M. L., 91
Barr, W. H., 387
Barrett, P. V., 246
Barrett, R. E., 210
Barriso, J., 149
Bartels, D. J., 317
Bartelstone, H. J., 406
Bartke, A., 267
Bartlett, D. J., 93
Bartter, F. C., 229
Baserga, R., 345
Bashore, R., 168
Bass, H., 125
Bass, J. W., 392
Bassett, C. A., 220, 225
Bassiri, R., 355, 356
Basu, D., 393
Batchelor, J. R., 138
Bates, D. V., 121, 125, 127
Bates, M. W., 273, 275-78
Bates, R. W., 251
Battaglia, F., 168
Battaglia, F. C., 168, 172
Bauer, H., 141
Bauke, J., 10
Baum, D., 63, 356
Baum, J., 57
Bauman, A., 328
Baumeister, A. A., 95
Baumgartner, H. R., 8, 9
Bayless, T. M., 105
Baylis, E. M., 398, 404
Bayrd, E. D., 77
Bazin, H., 85
Bazzano, G., 406
Bazzano, G. S., 406
Bealmear, P. M., 139
Bean, R. H. D., 78
Beardsley, J. V., 209, 212
Beaumont, J. L., 318
Beazley, J. M., 166, 169
Beck, J. S., 252
Becker, F. W., 198
Beckett, J., 77
Becklake, M. R., 124
Bedirian, K., 261
Beeley, L., 393
Beerel, F., 128
Behar, M., 103
Beilby, J. O. W., 135
Beischer, N. A., 167
Beisel, W. R., 19, 103
Bekker, F. J., 90
Bell, E. J., 145
Bell, J. J., 295, 297
Bell, R. G., 394
Bellanti, J. A., 141, 191
Beller, G. A., 405
Belmont, L., 94
Belzer, F. O., 86
Bendixen, G., 202
Benenson, A. S., 105
Ben Ezzer, J., 413
Bengtsson, E., 146, 148
Benjamini, E., 194
Benke, P., 49, 50, 54, 55
Benke, P. J., 58
Benkert, O., 211
Bennett, A., 26
Bennett, B., 177, 179, 202
Bennett, D. A. H., 373
Bennett, J. E., 193
Bennett, J. M., 197, 328
Bennett, M. J., 167
Bennett, P. N., 4, 8
Bennett, V., 237
Bennington, J. L., 348, 349
Bensch, V., 380
Bensen, W. W., 390
Benson, P. F., 13
Benyajati, C., 19, 103
Beran, A. V., 368
Berard, C. W., 329, 332
Berendes, H., 196
Berendes, H. W., 61
Berg, J. M., 91
Berg, J. W., 332
Bergentz, S. E., 7
Bergfors, P. G., 34
Berghoff, W., 278
Bergsagel, D. E., 282
Bergstrom, K., 148, 336
Berham, S., 141
Berk, P. D., 245, 247, 248
Berlin, A., 387
Berlin, N. I., 245-48
Berman, P. H., 42, 46-48, 52, 56
Bernanke, D., 374-76
Bernard, H. R., 294
Bernard, J., 12
Berndt, W. O., 42
Bershoff, S. N., 233
Bersohn, I., 316
Berson, S. A., 217, 220, 221, 223, 224, 226-28, 233, 234
Bertschinger, H. U., 107
Berzofsky, J. A., 35, 37
Beschi, R., 357
Besinger, T. A., 247
Besterman, E., 4, 9
Betuel, H., 191, 192
Bevan, C., 125
Bewley, T. A., 251
Bewsher, P. D., 233
Bhagwanani, S. G., 169
Bhalla, C. K., 58
Bhattacharya, S., 107
Bhoopalam, N., 201
Bieber, S., 57
Bieberdorf, F. A , 21
Bieg, A., 366, 379
Bieger, R., 397
Bienenstock, J., 85
Bierman, E. L., 319
BIGGAR, W. D., 135-44; 137, 138, 141
Bigger, J. T., 401, 406
Bignotti, G., 284
Bill, A. H., 202
Billewicz, W. Z., 167
Billings, B. H., 413
Binder, J. H., 22
Biörck, G., 3
Birch, H. G., 94, 95
Biro, Z., 192
Bishop, E. H., 167
Bitensky, M. W., 25
Bithell, T. C., 4
Bizarro, R. O., 63
Bizzozero, O. J. Jr., 75
Black, M., 413
Blackard, W. G., 310, 311, 313
Blackstone, M. O., 403
Blainey, J. D., 317
Blair, N. P., 21
Blakemore, W. S., 128, 241-43
Blanchley, P. H., 163
Blankenhorn, D. B., 319
Blatt, L. M., 237
Blau, M., 64
Bleicher, S. J., 221
Blinick, G., 162
Block, J. B., 331
Blomback, B., 335, 336
Blomgren, S. E., 401
Blond, D. M., 378, 379
Blonsky, R., 209, 212
Bloom, B. R., 176, 177, 179, 202
Bloomer, J. R., 246
Blume, R. S., 197
Bo, G., 6
Bobrow, M., 68, 72
Boder, E., 191
Bodmer, W. F., 68
Boesman, M., 175, 176
Boff, F., 212, 213
Boikan, W. S., 147
Bojesen, E., 366
Boler, J., 353
Bollman, J. L., 372
Bolton, C. H., 11
Bond, I. K., 92
Bond, V. P., 75, 345
Boneu, B., 10

Bonnadonna, G., 332
Bonnell, M., 321
Bonura, F., 37
Booyse, F., 4
Borchgrevink, C. F., 12
Borda, I. T., 393
Boreus, L. O., 32-34
Borga, O., 34, 398
Borgaonkar, D. S., 93
Borge, G., 214
Born, G. V. R., 13
Bortin, M. M., 138, 140
Bosch, H., 192
Bose, A. K., 107
Boshes, B., 209, 212
Bossak, E. J., 316
Botelho, S. Y., 123
Böttger, I., 304
Böttiger, L. E., 3, 11
BOUHUYS, A., 111-34; 111, 112, 115, 116, 120, 122-24, 126, 127
Boullin, D. J., 13
Boulton, J., 85
Boushy, S. F., 121
Bouvier, C. A., 9
Bowers, C. Y., 261, 353, 358, 359
Bowers, M. B. Jr., 211
Bowie, E. J. W., 2, 3, 5, 12
Boxer, L. A., 4, 10
Boxer, M. J. G., 4, 10
Boyd, A. E. III, 247
Boyd, E. M., 316
Boyd, R. J., 294
Boyes, R. N., 401
Boylan, J. W., 368, 374, 375
Boyle, I. T., 227
Boyle, J. A., 48, 51
Brace, K., 331
Bradbury, J. T., 168
Bradley, P. B., 214
Braemer, A. C., 106
Brain, M. C., 77
Braithwaite, R. A., 409
Braithwaite, T. A., 393
Brambati, B., 316
Bran, J. L., 86
Brand, C. R., 93
Brand, L., 389
Brandenburg, R. O., 63
Brandes, L. J., 203
Brandkamp, W. W., 92
Brandriss, M., 142
Brandriss, M. W., 200
Brauer, M. J., 78
Braun, K., 360
Braun, W. E., 341
Braunstein, G. D., 295-97, 299
Braunwald, E., 64
Breckenridge, A., 397
Breddin, K., 10
Breen, F. A., 340, 341
Breg, W. R., 68, 91
BRESLOW, J. L., 315-24
Brewer, H. B. Jr., 218
Bricker, N. S., 227
Bridger, G. P., 113
Bridges, J. M., 8
Bridges, R. A., 196
Bridson, W. E., 296
Brigden, W. D., 8
Brill, A. B., 75
Brink, G. A., 128
Briscoe, W. A., 125
Brittain, R. P., 93
Britten, R. J., 70
Brittinger, G., 72
Brodie, B. B., 30
Brodie, H. K. H., 209-12
Brodsky, I., 7
Brody, S., 11, 316
Broersma, R., 6
Bromberger-Barnea, B., 115, 116, 123, 128
Brook, J., 328
Brooks, R. V., 397
Brooks, S. M., 130, 414
Brosens, I., 167
Bross, I. D. J., 76
Brostoff, J., 179
Brown, A. K., 30
Brown, C. T., 368
Brown, D., 62, 141
Brown, D. F., 316, 317
Brown, E. G., 169
Brown, J., 350
Brown, J. B., 167
Brown, J. M., 79
Brown, W. G., 121
Brown, W. T., 141, 190
Bruce, R. A., 63
Bruce, W. R., 332, 350
Brumfitt, W., 86
Brunk, S. F., 325, 326
Bruns, P., 168
Brunton, M., 93
Bruton, O. C., 186
Bryan, J. A., 332
Bryant, G. D., 255
Buchanan, R. A., 398
Buchsbaum, M., 214
Buckingham, S., 4
Buckland, R. A., 70, 72
Buckle, R. M., 223, 225-29
Buckley, R. H., 138, 193
Buckner, C. D., 135
Buckton, K. E., 68, 70
Buescher, E. L., 191
Buhi, W. C., 167, 169
Buker, R. S. Jr., 62
Bull, J. M., 327, 331
Bullock, W. E., 142, 200
Bullock, W. E. Jr., 199
Bullough, W. S., 349
Bulot, G., 295
Buluk, K., 339
Bunney, W. E. Jr., 209-12
Bur, G. E., 92, 93
Burch, G. E., 145, 149
Burg, M., 366, 373
Burgee, S. L. Jr., 387
Burger, D. R., 176
Burgus, R., 353, 354, 357, 358
Burham, S., 62
Burke, C. W., 388
BURKE, J. F., 289-94; 289-91, 294
Burnet, F. M., 103
Burnett, C. H., 224
Burnett, H. H., 30
Burnett, W. S., 414
Burrill, K. C., 399
Burrows, B., 127
Burrows, W., 19, 21, 25
Burton, R., 34
Busch, G. J., 341
Bush, C. A., 406
Bush, M., 397
Butcher, L. L., 212, 213
Butcher, R. W., 26
Butler, J., 124
Butler, J. J., 327
Butler, P. S., 295
Butler, V. P. Jr., 403, 406
Butler, W., 128
Butt, W. R., 295
Bygdeman, S., 9
Byrnes, E., 67, 68

C

Cade, I. S., 135
Cade, J., 393
Caen, J. P., 12
Cahill, G. F. Jr., 303, 368-76
Calabresi, P., 77
Caldwell, J. H., 406
Caldwell, P. D., 93, 99
Calhoun, D. W., 11
Callahan, E. W. Jr., 241, 245, 247, 248
Callahan, J. A., 63
Callahan, W., 106
Calne, D. B., 210, 213
Cameron, J. L., 104
Cameron, J. S., 77
Campbell, B., 51
Campbell, D. C., 191
Campbell, D. J., 322
Campbell, E. J. M., 111, 128
Campbell, M., 61-63
Campbell, P., 30
Campbell, S., 169-71
Campbell, S. L., 166, 169
Campillo-Sainz, C., 63
Canales, L., 193
Canellos, G. P., 325, 328, 329, 331, 332
Canever, J. V., 8
Canfield, R. E., 295, 297
CANTERBURY, J. M., 217-32; 220, 221, 223-29
Caratzali, A., 72
Carbone, P. P., 295, 299, 325, 328

Cargille, C. M., 295
Carlson, L. A., 316
Carlsson, A., 212
Carmody, S., 407
Caro, C. G., 124
Carpenter, B., 223, 224, 226, 227
Carpenter, C. C. J., 19, 20, 26, 103-6
Carpenter, D. G., 49
Carpenter, J. W., 77
Carr, D. H., 91
Carr, J., 94
Carr, M. C., 183, 190
Carrel, R. E., 93
Carroll, W. R., 335, 339
Carter, C. H., 49
Carter, S. K., 327
Cartwright, G., 281-83
Casaer, P., 165
Casey, M. D., 93
Cash, J. D., 341
Cash, R. A., 105
Caspary, E. A., 11, 12
Caspersson, T., 67, 68, 72
Cassady, G., 168
Castaldi, P. A., 12
Castelli, W. P., 318
Castilla, E. E., 91
Castleman, B., 225
Catovsky, D., 78
Catt, K. J., 296, 297
Catz, C., 31, 32, 36
Caul, J., 7
Cavallin-Stahl, E., 242, 243, 246
Cavanaugh, J. H., 410
Celesia, G. G., 209, 210
Chadwick, A., 255
Chamberlain, D. A., 393
Champion, G. D., 392
Chan, T. L., 407
Chan, W. H., 169
Chandler, J. W., 193
Chang, J. K., 261
Chaparas, S. D., 195
Chaput de Saintonge, D. M., 77
Charnock, J. S., 378, 379
Charpin, J., 121
Chase, H. P., 194
Chase, L. R., 224
Chase, M. W., 176
Chason, J. L., 149
Chatterjee, B. D., 105, 106
Chatterjee, D. N., 103
Chatterjee, I. B., 31, 32
Cheifetz, D. I., 209-11
Chen, C. H., 316
Chen, L. C., 20, 22-24, 26, 104
Chen, T. R., 70
Cheney, F. W., 128
Chernay, P. R., 70
Cherniak, R. M., 129
Cherry, J. D., 146
Cherry, S. H., 168
Chertok, R. J., 368, 377
Chessells, J. M., 393
Chester, E. H., 112
Chevalier, R. B., 112
Cheyne, G. A., 167, 168
Chiang, S. T., 127
Chida, N., 316
Chiesara, E., 30
Chignell, C. F., 380, 388
Chilgren, R. A., 193
Chinard, F. P., 369, 370, 373
Chinoy, D. A., 379, 380
Chirito, E., 263
Chivot, J. J., 12
Chmielewski, J., 8
Choi, Y., 399
Choovivathanavanich, P., 79
Christensen, L. R., 336
Christian, J. C., 316
Chu, L. L. H., 219, 220
Chung, C. Y., 94
Chung, H. K., 85
Cifuentes DeLatte, L., 84, 86
Cintron, J., 3
Ciocco, A., 75
Cirnu-Georgian, L., 72
Clancy, R., 5
Clancy, R. L., 6
Clapham, W. F., 372
Clark, E., 2
Clark, G. R., 93
Clark, H. P., 145
Clark, R. L., 404
Clarke, P. S., 130
Clarke, S. W., 114, 119
Clarkson, A. R., 341
Clausen, J., 94
Clawson, C. C., 5
Cleaveland, C. R., 402, 403
Clément, J., 114, 115, 123
Clements, J. A., 169
Clemett, A., 188
Clemmons, D. R., 238
Cleveland, W. W., 141, 190
Close, H. G., 91
Cobbs, C. G., 84
COBURN, R. F., 241-50; 241-44, 246-48
Coccheri, S., 10, 13
Cockayne, E. A., 62
Coe, F. L., 229
Coen, L. J., 336
Cohen, A. I., 401
Cohen, B. D., 7
Cohen, H., 90
Cohen, J. J., 369-72
Cohen, M., 413
Cohen, R. J., 392
Cohen, T., 78
Cohn, D. J., 316
Cohn, D. V., 219, 220
Cohn, K. E., 327
Colcolough, H. L., 145, 149
Cole, A. P., 37
Cole, L. J., 138
Cole, W. R., 294
Colebatch, H. J. H , 116, 124, 127, 129
Coleman, M., 214
Colin, J., 316
Coller, B. S., 3
Collins, A., 246
Collins, M. H., 92
Collins, V. P., 281
Collombel, D., 44, 45
Collum, J., 93
Colman, R. W., 341
Coltman, C. A., 242, 243
Colton, T., 294
Combes, B., 310, 313
Comings, D., 72
Comroe, J. H. Jr., 111, 123, 125, 127
Condemi, J. J., 401
Condit, P. T., 350
Condoulis, W., 177
Cone, A. L., 277, 278
Conney, A. H., 36, 390
Connolly, C. K., 130
Connor, J. D., 49
Conolly, M. E., 402
Conrad, F. G., 79
Conradi, G., 322
Constantine, H. P., 129
Constantine, J. W., 13
Constantini, S., 366
Constantino, R. T., 401
Converse, J. M., 142
Cook, I. J. Y., 4
Cook, P. R., 53
Cooper, A. C., 77
Cooper, H. L., 389
Cooper, M. D., 80, 135, 191, 194
Cooper, R. G., 402
Cooperband, S. R., 179
Cope, O., 225
Copelan, H. W., 387
Corby, C., 147
Corcoran, J., 92, 93
Cordero, L., 168
Cornblath, M., 277, 278
Cornell, J. S., 297
Corneo, G., 72
Cornwell, A. C., 94, 95
Cornwell, J. G., 91
Corriere, J. N. Jr., 86
Corsello, P. R., 128
Corsen, S., 167
Cortes, E., 332
Corvisart, J. N., 147
Coscia, A., 361, 362
Costea, N., 201
Costom, B. H., 361
Cottier, H., 192
Cotzias, G. C., 210
Cournand, A., 125
Court Brown, W. M., 76, 79, 89-93, 95, 96
Courte, C., 358

Cowan, D. H., 7
Cowie, V., 89, 96, 97
Cox, C. E., 83-86
Cox, J. D., 332
Cox, P., 51
Crabbé, P. A., 85
Craddick, R., 93
Craig, J. M., 191
Craig, J. T., 105
Craig, L. C., 218
Craig-Holmes, A. P., 70
Crandall, B. F., 93
Crank, J., 117
Craver, L. F., 77
Crawford, G. A., 160
Creasman, W. T., 165, 167
Creditor, M. A., 228
CREGER, W. P., 75-82; 76, 78
Cremer, J., 278
Crigler, F., 36
Crofford, O. B., 234
Crombach, G., 211
Cronberg, S., 12
Cronholm, B., 408, 409
Cronkite, E. P., 75, 345
Cronquist, M., 8, 9
Crooks, J., 390
Crosby, W. H., 76
Crosignani, P. G., 316
Crosswhite, L., 285
Crounse, R. G., 346, 347
Crowley, J., 316
Crowley, J. M., 398
Crussi, F. G., 44
Crystle, C. D., 167
CUATRECASAS, P., 233-40; 233-39, 357
Culp, D., 84
Cumming, G., 112, 114, 117, 120, 122
Curd, J., 406
Curley, A., 413
Curlin, G. T., 26, 105
Curran, P. F., 22
Curtis, J. I., 35
Curzen, P., 167
Cushard, W. G., 228, 229
Cutler, S. J., 281, 284, 332
Czajka, D. M., 275
Czokalo, M., 339

D

Dacie, J. V., 77
Dagirmanjian, R., 212
Dahlquist, G., 275, 277, 278
Dahlstrom, A., 213
Daikuhara, Y., 237
Dalby, A. M., 8
Dallner, G., 31
Damasio, A. R., 209-11
Dameshek, W., 78, 282
Dammin, G. J., 341
Damus, P. S., 336
Dancis, J., 42, 46-48, 51, 52, 56
Danielson, G., 241
Danson, J., 126
Danta, G., 9, 10
Darlington, C. D., 70
Darmady, J. M., 316-18, 320
Darnell, A., 94
Dart, G. A., 114
Daughaday, W. H., 251, 255, 257, 259, 261, 264, 265
Davachi, F., 62
Davey, D. A., 167
David, J. R., 177-79, 193
Davidson, E. D., 391
Davies, M., 146
Davies, R. E., 368, 374, 375
Davis, C. W. C., 149
Davis, J. A., 168
Davis, J. C., 165
Davis, J. D., 93
Davis, J. M., 409
Davis, J. W., 7, 8, 10
Davis, L. E., 30, 31
Davis, S. D., 192
Davis, W. C., 195
Davison, K., 409
Dawson, A. M., 77
Dawson, B. F., 218
Day, H. J., 1
Day, R. W., 91
Dayman, H., 115, 122
Dayton, P. G., 389, 412
De, S. N., 103
Dean, A., 107
Deetjen, P., 368, 373
Deftos, L. J., 223
De Gaetano, G., 6
de Gasquet, P., 373
DEGRAFF, A. C. JR., 111-34; 121
de Grouchy, J., 61, 70
de Haseth, P. L., 68
Deicher, H., 198
de Jager, E., 296
de Jonge, H., 397
De Kock, M. A., 116, 129
de la Chapelle, A., 72
Delacretaz-Dubugnon, F., 11
de la Cruz, M. V., 63
Delamone, I. W., 78
deLange, D. J., 317
Delfs, E., 166
Delivoria-Papadopoulos, M., 242, 243, 246
Delobel, J., 12
del Pozo, E., 260, 261, 263
Deluca, H. F., 227
Delvoye, P., 255, 257
DeMars, R., 49-52, 54, 58
Dent, P. B., 197
DePasquale, N. P., 145, 149
Der Kaloustian, V. M., 49, 50
Desai, D., 332
DESBUQUOIS, B., 233-40; 234, 235
De Schaepdryver, A. F., 56
Desforges, J. F., 167
Desnick, S. J., 4
Desser, R. K., 325
Detter, J., 50
Dev, V. G., 70
DeVita, V. T., 325, 328, 329, 331, 332
de Vries, A., 283
de Wardener, H. E., 372
Dewhurst, C. J., 68
Dexter, R. A., 44
Deykin, D., 393, 394
Dhall, D. P., 6
Diamond, H. D., 77
DiBona, D., 24
Dicke, K. A., 137
Dicks-Mireaux, M. J., 94
Didisheim, P., 12
Diener, E., 196
Dierks-Ventling, C., 276-78
Dies, F., 369-73
DiGeorge, A. M., 42, 141, 190
DiGiacomo, J. N., 209-11
Dillaha, C. J., 351
Dincol, G., 79
Dincol, K., 79
Dingman, H. F., 94
Disalvo, J., 372, 373
DiScala, V. A., 368
Dixon, R. L., 31, 36
Djaldetti, M., 283
Doery, J. C. G., 1
Doherty, J. E., 403, 404
Dole, V. P., 154, 155, 157, 162
Doleschel, W., 316
Doll, R., 76
Dollery, C. T., 402
Dolovich, M. B., 117, 118
Domino, G., 95
Don, A. M., 92
Donald, I., 169, 170, 171
Donaldson, S. S., 325
Donati, M. B., 335
Done, A. K., 30, 392
Donnai, P., 167
Donnell, G. N., 54, 55
Doorenbos, D. E., 168
Doppman, J. L., 226
Dorfman, R. F., 325, 326, 331
Dormandy, K. M., 4
Dorward, W. J., 107
Dougan, L., 78
Douglas, A. S., 10, 13, 338
Douglas, S. D., 188, 189, 195
Dowben, R. M., 13
Doyle, J. T., 316, 317
Drabkin, D. L., 241

Drach, G. W., 84, 85
Drahota, Z., 278
Drakoff, I. H., 46, 56
Dray, S., 179
Dreifuss, F. E., 49, 57
Drets, M. E., 70
Dreyfuss, F., 10
Dreyfuss, J., 401
Drill, V. A., 11
Droegemueller, W., 11, 168
Drouin, G., 85
Dubach, U. C., 379, 381
Dube, B., 5, 12
Dubé, W. J., 223
DuBois, A. B., 123, 124, 128
Dubovsky, E., 357
Dubowitz, L. M. S., 172
Dubowitz, V., 172
Duckert, F., 6, 8, 9
Ducommun, P., 357
Dudley, G. M. III, 242, 243
Dudrick, S. J., 305
Duell, E. A., 349
Dufau, M. L., 296, 297
Duffell, G. M., 121, 124
Duffus, G. M., 169, 171
Dugdale, M., 11
Duggan, D. E., 378, 379
Duke, E., 390
Dumonde, D. C., 181
Duncan, C. L., 107
Du Plessis, J. P., 317
Dupont, A., 356
Dupont, B., 137
DUPONT, H. L., 103-10; 105
Dupont, R. L., 159
Dupré, J., 304
Durance, J., 120
Dutrilleaux, B., 70, 72
Dutta, N. K., 103
Dutton, G. J., 30, 32
Duvoisin, R. C., 210
Dymsza, H. A., 275

E

Eade, N. R., 396
Eaglestein, W. H., 351
Earley, L. E., 21
Eastman, N. J., 165
Ebadi, M. S., 392
Ebel, H., 24
Eckhardt, S., 284
Eder, H. A., 316
Edson, J. R., 4
Edwards, C. L., 326
Edwards, G. A., 77
Edwards, J. E., 62
Egdahl, R. H., 226-28
Eggleston, L. V., 377
Ehlers, K. H., 61
Eidelman, A. I., 168
Einthoven, W., 114, 115
Eisentraut, A. M., 303, 304
Eisner, E. V., 389
Eitzman, D. V., 193
Ekel, T. M., 346, 347
Ekert, H., 7
Ekins, R., 361
Eknoyan, G., 7, 8, 375-77, 379, 381
el-Baz, Z., 8
Elder, M. G., 166
el-Garf, T. A., 8
Elias, E. G., 284
Elion, G. B., 47
Elkeles, R. S., 11
Elkins, W. L., 137
Elliott, H. L., 19, 20, 26, 105
Ellison, J. R., 68
Ellman, L., 204, 213
Emmanuel, R., 61-63
Emmerson, B. T., 55
Empson, J., 93
Endler, N., 97
Endrenyi, L., 390
Engel, E., 93
Engel, J., 212, 213
Engel, R., 245, 247, 248
Engel, R. R., 244, 248
Engle, M. A., 61
Enns, T., 369, 370, 373
Epstein, B., 368, 377
Epstein, F. H., 374-76, 378-81
Epstein, L. J., 160
Epstein, R. B., 135, 140
Epstein, S., 214
Epstein, W. L., 345-48, 387
Erdem, S., 79
Erdheim, J., 252
Erhardt, A. A., 90
Ericsson, J., 32, 33
Erikson, U., 148
ERIKSSON, M., 29-40
Ernst, A. M., 212
Ernst, N., 321
Ertel, I. J., 413
Esselbaugh, N. C., 317
Estes, J. W., 393
Etherington, M., 6
Evans, C. A., 202
Evans, D. A. P., 390, 409
Evans, D. G., 19
Evans, H. J., 68, 70, 72
Evans, T. N., 167
Evens, R. G., 226
Everett, G. M., 212, 213
Ewy, G. A., 404
Eyssen, H., 85
Ezdinli, E. Z., 76, 285, 327, 332
Ezrin, C., 252, 253

F

Faelli, A., 366
Faglia, G., 361, 362
Fahey, J. L., 80
Fahimuddin, M., 105
Fahmy, D., 169
Fahn, S., 209-11
Fair, W. R., 84, 85
Fajardo, L. F., 327
Falek, A., 93
Falkow, S., 106
Fallatt, R., 319, 322
Faloona, G. R., 303-6
Fang, V. S., 222, 261, 357
Fanska, R. E., 36
Farber, P. A., 149
Farbiszewski, R., 9, 11
Farhi, L. E., 117
Farias, E., 149
Farnsworth, J. O. H., 84
Farnsworth, R. H., 84
Farquhar, J. W., 309, 314
Farr, R. S., 388
Farr, V., 172
Fawcett, J., 412
Fawcett, N., 54, 55
Feagin, O. T., 411
Fedoroff, S., 70
Feeley, J. C., 19
Fefer, A., 201
Feighner, J., 412
Feingold, M. L., 282
Feinleib, M., 284
Feldman, B. H., 85
Feldt, R. H., 63
Felig, P., 303, 369-73
Felix, J. S., 50, 51, 54, 58
Felsenfeld, O., 26
Felsenstein, J., 52
Fenn, W. O., 242
Ferguson, J., 91
Ferguson, J. H., 5
Ferguson-Smith, M. A., 89
Fernet, P., 10
Ferreira, H. C., 340
Ferris, B. G. Jr., 112, 114, 124
Feuer, G., 30
Fialkow, H., 128
Fichter, E., 35
Field, E. J., 12
Field, E. O., 80
FIELD, J. B., 309-14
Field, M., 19-23, 26, 104
Fields, J., 142
Fields, J. P., 200
Filley, G. E., 114
Finaz, C., 70
Finch, W. J., 1, 2
Finck, A. D., 26
Fink, D. J., 223, 224, 226, 227
Finkelstein, R. A., 19, 21, 104, 105
Finkelstein, S., 405
Finley, T. N., 128
Finnström, O., 172
Finsberg, L., 413
Finstad, J., 135
Finucane, K. E., 124
Fiorentini, F., 10

Fiorentini, P., 13
Fireman, P., 175, 176
Firkin, B., 5
Firkin, B. G., 6
Fisch, S., 397
Fischer, J. J., 325, 326
Fish, C. H., 42
Fish, L. A., 197
Fisher, A. B., 123
Fisher, D. A., 356
Fisher, F., 335, 336, 339
Fisher, J., 222
Fisher, R., 146
Fisher, R. E., 84, 86
Fishman, J. R., 92
Fitzgerald, D. E., 10
Fiume, L., 32
Flanigan, W. J., 404
Fleischer, N., 358, 361, 362
Fleischer, S., 276
Fleming, J. S., 366-68
Flessa, H. C., 393
Fletcher, A. P., 335-39
Fletcher, C. M., 127
Fliedner, T. M., 345
Fliegner, J. R. H., 165
Flocks, R. H., 84
Flores, M., 316
Flouret, G., 353
Floyd, W. S., 167
Fogel, B. J., 141, 190
Fogelman, A. M., 405
Foley, H. T., 327
Foley, T., 361
Folger, G. M. Jr., 62
Folk, I. E., 339
Folkers, K., 261
Folley, S. J., 255
Fomon, S. J., 317
Foraker, A. G., 121, 127
Ford, S., 319, 322
Fordtran, J. S., 21
Forgione, H., 326
FORMAL, S. B., 103-10; 103, 106, 107
Forman, W. B., 391
Forrest, F. M., 408
Forrest, I. S., 408
Forsham, P. H., 399
Forssman, H., 89, 92
Forster, R. E., 241-44, 246
Forstner, G. G., 24
Forsyth, I. A., 255
Forte, L. R., 366, 378, 380
Fosbrooke, A. S., 316-18, 320
Foulkes, D. M., 403
Fournier, A. E., 227, 223
Fournier, M. R., 83
Fouts, J. R., 30, 31, 36
Fowler, W. S., 125
Fox, I. H., 48, 49
Fox, M. H., 63
Fox, R., 165
Franchimont, P., 295, 299
Francis, S. H., 94
Francke, U., 51
Franckson, J. R. M., 233
Frantz, A. G., 224, 252, 255-59
Frantz, W. L., 255
Franzén, S., 202
Fraser, F. C., 61
Fraser, R. B., 116
Fraser, R. G., 121
Fraumeni, J. F. Jr., 78, 80
Frayser, R., 129
Frazer, G. R., 52
FREDRICKSON, D. S., 315-24; 316-22
Freedland, R. A., 374
FREEDMAN, D. X., 153-64; 162
Freeman, C., 332
Freeman, T., 316, 317
Fregni, L., 10
Frei, E., 348, 349
Frei, E. III, 331
Freinkel, N., 356
Frenzel, Y., 35, 37
Freychet, P., 234, 235, 237
Frick, P. G., 5
Fridman, M., 326, 327
Friedberg, N. M., 1, 3
Friedlander, I., 4
Friedman, G., 317, 320
Friedman, M., 332
Friedman, R., 57
Friedmann, T., 52
Friend, J., 167
FRIESEN, H., 251-70; 251-55, 257-63, 266, 357
Frindel, E., 348, 349
Fris-Hansen, B., 317
Frøland, A., 92
Fromm, D., 104
Frommeyer, W. B. Jr., 283
Frontino, G., 30
Frost, P., 345, 346, 348-51
Fry, D. L., 115, 116, 119, 128, 129
FUDENBERG, H. H., 175-208; 12, 142, 175, 177, 178, 182, 183, 188-90, 193-96
Fuhrman, F. A., 104
Fuhrman, G. J., 104
Fuhrmann, W., 61, 62
Fujimoto, M., 366-69, 376, 379
Fujimoto, W. Y., 51, 57
Fujita, H., 89, 90
Fujita, T., 222, 228
Fukuyama, K., 347
Fulgraff, G., 366, 379
Fuller, L. M., 76, 327, 329
Fulton, J. E. Jr., 348
Funk, C., 340
Furlaneto, J. A., 127
Furman, M., 36
Furth, J., 265
Fusaro, R., 142
Fuxe, F., 213, 259

G

Gabica, J., 390
Gall, J. G., 70
Gallagher, D. J. A., 84
Galloway, L. S., 317
Gallus, A., 393
Galton, D. A. G., 78, 203, 281, 282, 284
Gamble, J. F., 327, 329
Gams, R. A., 79
Gamsu, G., 118, 119, 121
Gangarosa, E. J., 19, 103
Ganguly, U., 106
Ganner, E., 68
Garabedian, M., 227
Garattini, S., 398
Garb, S., 385
Garcia, A. P., 378
Garfield, D. H., 78
Garratt, C. J., 233
Garrett, W. J., 170
Garron, D. C., 89, 90, 209-11
Garson, O. M , 91
Gartler, S. M., 50, 51
Gartner, L. M., 36, 37, 413
Gascoyne, T., 373
Gassanein, A. A., 8
Gatmaitan, B. G., 149
Gatmaitan, Z., 36, 388
Gatti, R. A., 136, 141
Gautier, M., 67
Gavin, J. R. III, 234
Gaynor, E., 12
Gayrard, P., 121
Geary, C. G., 76, 78
Gehan, E. A., 327
Gelborn, H. V., 389
Gelfand, M. M., 316
Gellhorn, A., 281
Gemski, P., 107
Genant, H. K., 379
Gentz, J., 316, 317
Genuth, S. M , 227
Georg, J., 117
George, R. P. Jr., 76
Geormaneanu, C., 72
Gerard, P. W., 146
Gerard-Marchant, R., 332
Gerber, N., 397
Gerhardt, R. F., 400
Gershanik, J. J., 141
Gershon, E., 20, 22, 23, 26, 104
Gershon, S., 407
Gerson, C. D., 398
Gertner, M., 68
Getz, M. J., 72
Gevers, W., 354
Ghadimi, H., 58
Giblett, E. R., 135
Gibson, D., 94
Gibson, L. E., 120, 126
Giebisch, G., 366, 368, 379

Giknis, F. L., 63
Gilchrist, G. S., 7
Gillenwater, J. Y., 84
Gillette, J. R., 31
Gillie, E., 316, 317
Gililand, J. C., 61
Gilman, A., 156
Gilmore, E., 407
Gindilis, V. M., 91, 92
Gingerich, R. L., 310, 313
Ginsburg, A. D., 1
Gitlin, D., 175, 176, 191
Givler, R. L., 325, 326
Gjerdrum, K., 405
Glade, P. R., 198
Gladner, I. A., 339
Gladtke, E., 34
Glanzmann, C., 360
Glasgow, L. A., 149
Glatstein, E., 325, 326
Glazko, A. J., 35
Glew, R. H., 105
Glidewell, O., 328, 329
Glinsukon, T., 26
Glorieux, F., 228
Glover, M. K., 326, 327
Glowacki, G. A., 168
Gluck, L., 168, 169
Glueck, C. J., 316, 319, 320, 322
Glueck, H. I., 7, 8
Gmür, J., 340
Goalwin, A., 317
Gocka, E. F., 328
Godal, H. C., 12
Godal, T., 179
Godfrey, H. P., 195
Godolphin, W. J., 322
Gold, M., 369
Gold, N. I., 36
Gold, W. M., 116, 124
Goldberg, C., 172
Goldberg, L. M., 83
Goldberg, N. B., 104
Goldberg, N. O., 20
Goldberg, S. J., 317, 320
Goldenfarb, P. B., 10
Goldfinger, D., 146, 148
Goldfinger, S. E., 403
Goldie, W., 191
Goldman, J. M., 329
Goldman, L., 378
Goldmann, J., 316
Goldring, D., 34
Goldsmith, H. L., 3
Goldsmith, R. S., 220, 221, 223
Goldstein, A., 159, 163
Goldstein, J. L., 50, 51, 319
Goldstein, M. L., 12
Goldstein, M. S., 260
Goldstein, S., 52
Goluboff, L. G., 252, 253
Gomprecht, R. F., 397
Gonda, A., 263
Gonick, H. C., 380
GOOD, R. A., 135-44; 80, 135-38, 141, 142, 191, 193, 194, 196, 197
Goodell, H., 209, 212
Goodman, L. S., 156
Goodman, P. A., 167
Goodwin, F. K., 209-12, 214
Goodwin, J., 63
Gorbach, S. L., 105-7
Gordan, G. S., 223, 224, 226, 227
Gorden, P., 237, 251
Gordon, E. E., 381
Gordon, H., 167
Gordon, J., 178, 181
Gordon, J. E., 103
Gordon, M. B., 316
Gordon, R. S., 104
Gordon, T., 318
Gorlin, R. J., 64
Gorman, R. E., 25
Gormsen, H., 146
Gosh, A. K., 107
Gotoff, S. P., 179
Gotsman, M. S., 149
Gotterer, G. S., 24
Gottlieb, C. W., 221
Gottlieb, J. S., 13
Gottstein, U., 278
Gould, L., 397
Goulding, R., 409
Goulet, C., 9
Goverde, B. C., 296
Grabar, P., 336
Grace, J. T. Jr., 286
Grady, F. G., 107
Graham, B. A., 8
Graham, T. C., 140
Graham, W., 123
Gram, L. F., 413
Granger, G. A., 179
Granoff, D., 89, 90
Grant, G., 357
Grant, R., 1, 2
Grave, G. D., 278
Graven, S. N., 169
Graves, L., 388
Gray, C. H., 241
Grayburn, J. A., 314
Grayer, D. T., 26, 105
Green, G. M., 84
Green, I., 204, 213
Green, J. A., 179
Greenberg, B. G., 317
Greenberg, N., 90
Greenberg, S. D., 121
Greenberger, N. J., 406
Greene, F. E., 409
Greene, M. L., 48, 50, 52, 54, 55, 57
Greene, R., 126
Greenough, W. B. III, 19-21, 26, 104-6
Greenwald, P., 414
Greenwood, F. C., 220, 234
Greulich, R. C., 347
Greves, J. H., 316
Griffiths, A. W., 93
Grifoni, V., 284
Grimby, G., 123
Grindlay, J. H., 372
Griner, P. F., 396
Griscelli, C., 191, 192
Grist, N. R., 145
Grodsky, G. M., 36
Groh, R. H., 209-11
Grossman, S. P., 355
Grossmann, H., 265
Gröttum, K. A., 4, 193
Gruenwald, P., 170, 172
Grumbach, M. M., 295, 361
Gryboski, J. D., 188
Gual, C., 353, 358, 360, 361
Guansing, A. R., 357
Guerrant, R. L., 22, 26, 106
Guichard, A., 401
Guillemin, R., 353, 354, 357, 358
Guiraud, B., 10
Gulesserian, H. P., 325, 326
Gunne, L.-M., 211
Gunz, F. W., 76
Gurewich, V., 340, 341
Gusdon, J. P., 169
Gustavson, K. H., 93
Gutman, A. B., 389
Gutterman, J. V., 77
Guyda, H., 251-54, 257-61
Gydall, K., 12
Gyermek, L., 30
Gyles, C. L., 106

H

Haas, J. E., 145
Habbu, M. K., 103
Habener, J. F., 219-22
Haber, E., 405, 406
Hackeng, W. H. L., 223, 224, 228
Haddad, Z. H., 175, 176
Hadidi, T., 77
Hague, D. E., 396
Hague, N., 399
Hahn, P., 278, 316
Haigler, D. E. Jr., 358-61
Haining, R. G., 399
Halgrimson, C. G., 79
Hall, J. G., 62
Hall, M. S., 404
Hall, R., 353, 362
Hall, S. M., 98
Hall, W. T., 202
Halliday, W. J., 202
Halls, S., 106
Halprin, K. M., 347
Halvorson-Politis, E., 316
Ham, J. M., 11, 13
Hambert, G., 92
Hamerton, J. L., 91
Hames, C. G., 317

Hamilton, J. W., 219, 220
Hammaker, L., 244
Hammar, C. G., 408
Hammond, D., 7
Hammond, W. G., 226, 229
Hampson, J. C., 90, 91
Hampson, J. L., 90, 91
Hampton, J. R., 1, 3, 5, 6, 8, 10, 11, 13
Hamstra, R., 120
Han, T., 332
Hancock, E. W., 327
Hancock, P. E. T., 331
Hand, W. L., 85
Handlon, S., 295
Hanks, G. E., 332
Hanley, H. G., 86
Hansen, G. S., 137
Hansen, H. H., 332
Hansen, J. M., 397
Hanson, J. F., 146
Hansten, P. D., 385
Hard, M. M., 317
Harding, H. B., 145
Hardisty, R. M., 2-4
Hardy, J., 252, 259, 263
Hargreaves, T., 37
Harker, L. A., 1
Harlan, W. R. Jr., 321
Harnden, D. G., 91
Harper, D. T., 105
Harrah, J., 380
Harris, A., 146
Harris, D. A., 167
Harris, H., 53
Harris, J., 77, 95, 97
Harris, R., 147
Harrison, D. C., 401
Harrison, E. G. Jr., 76
Harrison, M. J. G., 3
Hart, L. G., 36
Hartdegen, R. G., 228
Hartmann, G., 9
Hartree, A. S., 297
Hass, A. C., 325, 326
Hassanein, A. A., 338
Hathaway, W. E., 13
Hattler, B. G., 193
Haugaard, N., 233
Haugen, O. A., 252
Hauschild, A. H. W., 107
Haut, A., 281-83
Hawiger, J., 340
Hawk, R. E., 413
Hawkey, C., 4
Hawkins, C. F., 392
Hawkins, D., 388
Hawkins, R. A., 273, 275-78
Hawkins, R. L., 1
Hayashida, T., 252
Hayes, A., 402
Hayes, R. L., 326
Hayes, T. L., 84-86
Hayter, C. J., 372
Hazzard, W. R., 319
Heady, J. A., 10
Heath, C. W. Jr., 77, 78
Heath, H., 8
Heckman, F., 316, 320
Heckscher, T., 125
Hedner, U., 341
Hedrick, S. R., 195
Heffelfinger, J. C., 398
Heidenrich, R. O., 5
Heinonen, J., 402
Heizen, W. H., 403
Held, K., 278
Helin, M., 148
Hellegers, A., 168
Hellegers, A. E., 166
Hellem, A. J., 1, 3, 5, 8
Heller, J. H., 89, 94
Heller, P., 201
Hellman, L. M., 169, 171
Hellström, I., 139, 202
Hellström, K. E., 139, 202
Helmholz, H. F. Sr., 83
Helmken, J., 91
Helpern, M., 146
Hempelmann, L. H., 76
Hems, R., 377
Henderson, A., 20, 22, 23, 26, 104
Henderson, J. A., 117, 118, 121
Henderson, J. F., 50, 54, 55
Henderson, P. T., 30
Hendler, E., 379, 381
Hendler, R., 303
Hendrix, T. R., 26, 105
Heninger, G., 211
Hennen, B. K. E., 42
Henriksson, P., 7
Henry, G. M., 209, 212
Hep, K. D., 239
Herbert, D. C., 252
Herbert, P., 321
Herbert, V., 221
Herbst, A. L., 414
Herdman, R., 85
Heremans, J. F., 85
Herlant, M., 252, 253
Hermier, M., 44, 45
Herms, W., 368
Hernandez, A., 34
Herold, R., 340
Herr, H. M., 93
Herrera, J., 369, 370, 372, 373
Herrick, N., 54, 55
Herrmann, E., 315
Herrmann, I., 220, 225
Hershman, J. M., 353, 358, 360, 361
Herskovic, T., 188
Hertzog, H., 120
Herzenberg, L. A., 138
Herzig, R. H., 336
Herzog, C., 191, 192
Hewitt, D., 76
Heyssel, R. M., 75
Heywood, J. B., 9, 10
Hicken, P., 120
Hickman, J. W., 296
Higgins, J. T., 21
Hilgenberg, F., 61
Hill, A. B., 76
Hillarp, N. A., 213
Hillman, C. C., 233
Hills, A. G., 233
Hines, J. D., 7
HINMAN, F., 83-88; 83-86
Hintz, R. L., 238
Hira, N. R., 83
Hirsch, S. R., 92
HIRSCHHORN, K., 67-74; 68, 70
Hirschhorn, N., 24, 105
Hirschhorn, R., 72
Hirschman, R. J., 6
Hirsh, J., 1, 9, 393
Hiscox, J. L., 44
Hitchings, G. H., 57
Hitzig, W. H., 192
Hoagland, R. J., 146
Hobbs, J. R., 77, 193
Hobson, B., 296
Hodges, R. G., 317
Hoefnagel, D., 42
Hoehn, M. M., 210
Hoenig, J., 92
Hoffman, A. A., 83
Hogen, T. C., 263
Hogg, J. C., 120
Hognestad, J., 6
Hohenauer, M., 35
Hohenleitner, F. J., 369, 370
Hökfelt, T., 259
Holcomb, R. W., 75
Holgate, H., 353
Holick, M. F., 227
Holik, K., 107
Holland, J. F., 78, 332
Hollander, C., 359, 360
Holliday, M. A., 380
Holman, G. H., 35
Holmes, B., 196
Holmes, E. C., 202
Holmes, E. W., 368
Holmsen, H., 1, 193
Holmstedt, B., 408
Holt, L., 193
Holt, M., 63
Homan, J. D. H., 296
Hong, R., 85, 136, 188, 193
Hong, S. K., 368
Hood, W. B. Jr., 405
Hooft, C., 56
Hoogstraten, B., 327, 332
Hook, J. B., 379, 380
Hope, K., 93
Horn, A. S., 214
HORNICK, R. B., 103-10
Hornung, R., 316
Hornych, A., 21
Hornykiewicz, O., 213
Horowitz, H. I., 3, 7, 8
Horrobin, D. F., 259

Horsfield, K., 114, 117, 120, 122
Horst, W., 360
Horton, J., 328
Horvath, I. P., 284
House, B. J., 96
House, P. D. R., 234
Houser, J. J., 393
Houts, P., 98
Hovig, T., 193
Howard, J. G., 138
Howard, P., 127, 162
Howard, R. S., 41, 45
Hower, R. B., 245, 247, 248
Howes, J. F., 392
Hsia, D. Y., 13
Hsieh, Y. C., 129
Hsiung, G. D., 146
Hsu, L. Y., 68, 70
Hsu, T. C., 67, 70-72
Huber, G. L., 128
Huber, G. S., 104
Huber, H., 195
Hudson, R. E. B., 145, 148
Hughes, E. A., 167
Hughes, L. E., 202
Hughes, P. H., 160
Huisjes, H. J., 167
Hulet, W. H., 368, 377
Hulten, M., 68
Humair, L., 341
Hume, D. M., 79, 80
Hummeler, K., 192
Humphreys, M. H., 21
Humphreys, R. E., 395
Hunt, D., 64
Hunt, J., 97
Hunt, L. B., 112
Hunt, V., 12
Hunt, V. R., 122, 123, 126
Hunter, F. E., 371
Hunter, G. Jr., 246
Hunter, J., 399
Hunter, W. H., 392
Hunter, W. M., 220, 234
Huntley, C. C., 62
Huq, M. I., 105
Hurley, W. P., 93
Hurwitz, A., 385
Huser, H. J., 192
Hutchinson, D., 120
Hutchinson, E. C., 9
Hutchison, G. B., 76, 326
Huth, K., 9
Hutton, R. A., 2-4
HWANG, P., 251-70; 251-54, 257-61, 263
Hyatt, R. E., 114-16, 122, 123, 126, 128, 129
Hyde, R. W., 123
Hynie, S., 20, 22, 23, 26, 104

I

Iatridis, P. G., 5
Iber, F. L., 21, 26, 104, 105
Idanpaan, J., 34
Ide, T., 311, 313
Illiano, G., 234, 236, 239
Immergut, M., 84
Inaba, T., 390
Ingram, R. H., 121, 124, 125
Institoris, L., 284
Irie, M., 360
Irvine, R. A., 296
Ishikawa, S., 129
Ishimaru, T., 75
Islam, M. R., 105
Israel, H. L., 199
Israëls, M. C. G., 77, 283
Isselbacher, K. J., 24
Itabashi, H. H., 44
Itoh, T., 277, 278
Itscoitz, S. B., 1
Iyengar, R. N. V., 165

J

Jablon, S., 76
Jacina, J., 316
Jacks, T. M., 106
Jackson, C., 168
Jacobs, B., 105, 106
Jacobs, L. S., 255, 257 259, 261, 264, 265, 359
Jacobs, P. A., 91, 92
Jacobson, C. B., 51
Jacoby, H. I., 26
Jaeger, M. J., 114
Jaffe, J., 159, 162
Jaffe, J. H., 163
Jaffe, R. B., 295
Jahn, C. L., 146
Jalling, B., 34
James, J., 9
James, J. A., 56
James, R. H., 127
James, R. L., 96
Jamieson, S., 6
Jammet, M. L., 190
Janovsky, M., 168
Jarett, L., 251
Jarho, L., 402
Jarrett, R. J., 233
Jaslowitz, B., 77
Jaton, J. C., 406
Jeffcoate, T. N. A., 165
Jelliffe, A. M., 135
Jelliffe, R. W., 404, 405, 414
Jelliffe, S. M., 414
Jen, P., 234
Jenkins, D. E., 121
Jenkins, E., 5, 6
Jenkins, R. B., 209-11
Jensen, E., 266
Jensen, J., 319
Jensen, O. N., 398
Jenson, C. B., 294
Jerec, E., 162
Jeremic, M., 193
Jeremy, D., 79
Jerushalmy, Z., 1, 2, 6
Jervell, J., 405
Jessop, J. D., 77
Jeter, W. S., 176
Jeune, M., 44, 45
Jick, H., 128, 393, 412
Jiorg, N., 295
Jockes, A. M., 403
Joffe, B. I., 228
Johannesen, J., 367
Johansson, B. W., 63
Johansson, C., 67
Johansson, M. B., 242, 243, 246
Johnsen, S. G., 92
Johnson, A. J., 339
Johnson, G., 186
Johnson, G. E., 390
Johnson, J., 20, 22, 23, 26, 104
JOHNSON, J. W. C. JR., 165-74
Johnson, K. G., 75
Johnson, R. A., 178, 181
Johnson, R. C., 94
Johnson, R. E., 325-27, 332
Johnson, R. L. Jr., 121
Johnson, W. J., 228
Jonasson, J., 68
Jondorf, W. R., 30
Jones, E., 321
Jones, E. M., 392
Jones, G. E. S., 166
Jones, J. G., 114, 116, 117, 119, 122
Jones, J. M., 139
Jones, J. W., 316
Jones, K. W., 72
Jones, M., 13
Jones, N. L., 127
Jones, S. E., 327, 331
Jones, V. D., 381
Jonson, B., 123, 124
Joo, P., 140
Jorgenson, P. L., 380
Jose, D. G., 139
Jouppila, P., 32, 33
Jowsey, J., 228
Jubiz, W., 223
Juchau, M. R., 31
Judd, L. J., 92
Jude, J. R., 372
Jurman, M., 27
Jusko, W. J., 388
Justice, P., 13

K

Kadar, D., 390
Kadin, M. E., 325, 326, 331
Kagnoff, M. F., 3
Kahn, L. B., 329
Kahn, R., 234, 235
Kahn, S. B., 7, 241, 246,

248
Kalden, J. R., 198
Kaliss, N., 202
Kalow, W., 390
Kamimoto, T., 272
Kamin, R., 189
Kammerman, S., 295, 297
Kamoun, P., 8
Kampffmeyer, H. G., 392
Kanazawa, Y., 311, 313
Kane, P. B., 244, 247
Kaneko, K., 117, 118
Kaneshiro, M. M., 1, 2
Kang, K. W., 316
Kaniecki, E. A., 105
Kannegiesser, H., 376
Kannell, W. B., 318
Kanter, A., 227
Kao, V. C. Y., 25
Kapadia, G. G., 404
Kaplan, A., 316
Kaplan, A. R., 91, 97
Kaplan, H., 194
Kaplan, H. S., 80, 325-28, 331
Kaplan, N., 309-11
Kaplan, S., 61
Kaplan, S. L., 295, 361
Kapp, J. P., 9
Karaca, M., 13
Karam, J. H., 399
Karchmer, A. W., 105
Kardish, R., 30
Karki, N. T., 32, 33, 37
Karlberg, B., 360, 361
Karnovsky, M. L., 179, 196
Karpatkin, S., 3, 6
Karshan, M., 317
Karzmark, C. J., 327, 328
Kass, E. H., 84
Kastin, A., 353, 358, 360, 361
Kasturi, J., 12
Kato, H., 72, 76
Kato, R., 30, 31
Katurich, N., 368
Katz, A. I., 378-80
Katz, R. L., 26
Kaufman, H. S., 124
Kaufman, J. M., 52
Kaufman, L., 245
Kaufmann, J. S., 411
Kawai, C., 149
Kawasaki, S., 75
Kay, H. E. M., 138, 190, 192
Kay, J. L., 57
Kaye, D., 84, 86
Kean, E. L., 368, 374, 375
Keast, D., 139
Kedracki, M., 316
Keele, D. K., 49, 50, 57
Keen, H., 233
Keenaghan, J. B., 401
Keeri-Szanto, M., 390
Kelley, W. N., 41, 45-50, 54-56
Kelly, J. A., 170
Kelly, P., 261
Kelly, P. J., 228
Kelly, S., 94
Kemp, D., 13
Kempe, C. H., 200
Kemper, B., 219
Kendall, B. E., 226
Kendall, M. J., 392, 393
Kennedy, B. J., 283
Kennedy, C., 278
Kennedy, J. F., 353, 354
Kennedy, M. S. C., 112, 130
Kerridge, D. F., 172
Kerstein, L., 263
KESSLER, R. H., 365-84; 366-69, 375-78, 381
KESSLER, S., 89-102; 93, 96, 97
Ketcham, A. S., 202
Kety, S. S., 271-73, 275, 276, 278
Keutmann, H. T., 218
Key, B. J., 214
Keynes, W. M., 225
Khan, C. R., 237
Khanna, N., 37
Khazin, A. F., 168
Khurani, C. M., 107
Kibrick, S., 179
Kiely, J. M., 76
Kihlborn, M., 90
Kiil, F., 366, 367, 378, 379, 381
Kikkawa, Y., 168
Kilbourne, E. D., 149
Killenberg, P. G., 391
Kim, B. M., 122, 123, 126
Kim, K. H., 237
Kim, U., 76, 327
Kimball, H. R., 327
Kimberg, D. V., 20, 22, 23, 26, 104
Kimbrough, R. D., 413
Kimura, I., 332
Kimura, K., 212
Kincaid-Smith, P., 6
Kinsky, R., 138
Kirchenbaum, D. M., 58
Kirkpatrick, C. H., 178, 181
Kishan, R., 12
Kisieleski, D., 4
Kittrich, M., 168
Kiviniemi, K., 286
Kivowitz, J., 92, 93
Kizer, D. E., 272
Kjekshus, J., 367
Klawans, H. L. Jr., 211
Kleber, H. D., 162
Klee, C. B., 277
Kleeberg, J., 316
Klein, E., 202
Klein, I., 26
Kleinberg, D. L., 252, 256, 257, 259
Kleinkauf, H., 354
Kleinrok, Z., 212
Kleinzeller, A., 378
Kligerman, M. M., 325, 326
Kligman, A. M., 347
Kline, I. K., 147
Klinger, W., 35, 37
Klose, R. M., 368, 375, 379
Knight, J., 112, 121
Knochel, J. P., 304
Knotkova, A., 378
Knouss, R. F., 400
Knox, F. G., 366-68
Knudson, R., 114
Knudson, R. J., 129
Kobari, K., 105
Kobayashi, I., 10
Koch, Y., 259
Kochwa, S., 1, 2, 168
Koch-Weser, J., 388, 391, 393, 395, 396, 400, 403, 407
Kockott, G., 211
Kogut, M. D., 54, 55
Kohl, M. S., 210, 212
Kohler, E. M., 106
Kohler, P. O., 296
Kohn, G., 91
Kohne, D., 70
Kolb, H. J., 36
Kolb, W. P., 179
Kolkovsky, O., 316
Komoras, M., 316
Komp, D. M., 7
Konno, S., 145
Kono, T., 234, 236
Kopeć, M., 6, 337
Kopin, I. J., 409
Korc, I., 316
Korhonen, O., 130
Kornerup, V., 317
Korones, S. B., 61
Korsan-Bengtsen, K., 11
Kosaka, K., 311, 313
Koss, L. G., 282
Kowalski, E., 337, 338, 340
Krajny, M., 406
Krakauer, R. S., 140
Kralovanszky, J., 284
Kramer, K., 368, 373, 374
Krasner, J., 31, 32
Krauss, R., 321
Kraut, J. W., 327
Krebs, H. A., 273, 275-78, 370-73
Kreek, M., 154, 155, 157
Krejci, J., 179
Kremer, W. B., 138
Krenitsky, T. A., 47
Krens, K. E., 130
Kretschmer, R., 62, 141
Krill, C. E. Jr., 244
Krivit, W., 194
Krompotic, E., 146, 149
Kronman, B. S., 202
Krug, F., 234, 235, 238

Krug, U., 234, 238
Krull, P., 198
Krumholz, R. A., 112
Kruskemper, H., 361
Kuba, K., 9
Kubisz, P., 6, 12
Kübler, W., 35
Kubo, T., 372
Kubota, Y., 107
Kuesch, G. T., 107
Kugel, R. B., 392
Kullander, S., 11
Kuller, L., 146
Kulovich, M. V., 168
Kumahara, Y., 361
Kumar, P. J., 77
Kundel, D., 103
Kunev, N., 103
Kunimasa, I., 332
Kuntzman, R., 36, 390
Kuntzman, R. G., 389
Kuramoto, A., 4
Kurland, L. T., 63
Kurnick, J. E., 286
Kurowska, T., 11
Kutt, H., 397, 398
Kuze-Capar, Z., 146
Kuzuya, T., 311, 313
Kvale, J. N., 92
KWAAN, H. C., 335-44; 336, 338, 341
Kwiterovich, P. O., 316-20, 322
Kyle, R. A., 77, 282

L

Labbe, R. F., 399
LaBrec, E. H., 103
Labrie, F., 357, 358
Labrune, B., 190
LaChapelle, J. M., 346
Lachmann, P. J., 179
Lackner, H., 12
Lacko, A. G., 316
LaCour, L. F., 70
Lacy, S. S., 83
Laki, K., 339
Lalezari, P., 339
Lamberger, B., 148
Lambrew, C. T., 64
La Mont, J. T., 405
Lamvik, J. O., 179
Lamy, M., 61, 190
Landaw, S. A., 241, 245, 247, 248
Landberg, T., 327
Landes, R. R., 83
Landing, B. H., 196
Landon, E. J., 366, 378, 380, 381
Landon, M. J., 168
Landwehr, D. M., 379
Lapinleimu, K., 148
Lapointe, N., 140, 141
Lapp, N. L., 122
Laron, Z., 316
LaRosa, J. C., 321
Larrieu, M. J., 6, 336, 339
Larson, W., 91
Larsson, S. O., 7, 8
Lasch, H. G., 9
Laskowski, D. S., 316
Lassen, N. A., 117, 366, 367
Laszlo, J., 77
Latallo, Z. S., 6, 337, 338
Lau, K. S., 221
Laugier, A. J., 332
Laurence, D. R., 210
Laurence, E. B., 349
Laurent, T. C., 336
Lauritzen, C., 295
Laverty, R., 212
Law, L. W., 80
Lawrence, A. M., 263
Lawrence, H. S., 142, 175-77, 179, 199, 203
Lawrence, J. H., 77
Lawrence, J. S., 75
Lawrence, R. A., 167
Lawton, N. F., 361
Lazarus, S., 116
Leatham, A., 146
Leavitt, F., 209-11
LeBlanc, P., 126
Leblond, C. P., 347
Lecker, D. L., 199
Lecompte, F., 9
Ledlie, E. M., 331
Lee, B. J., 332
Lee, C. W., 146
Lee, C. Y., 297
Lee, H. M., 79, 80
Lee, J. B., 224, 368, 373-76, 379
Lee, J. C., 85
Lee, J. E., 209, 212
Lee, J. G., 346, 347
Lee, R. V., 62
Lee, S. G., 295
Lee, V. F., 316
Lees, R. S., 317, 318
Lefebvre, E. B., 399
Lefebvre, P. J., 303
Lefkowitz, R., 357
Lehmann, W. D., 295
Lehninger, A. L., 276, 277, 369
Leipold, W. D., 96
Leitch, G. J., 21, 26
Leith, D. E., 114, 118, 119, 121
Lejeune, J., 67, 70, 72
Lejnieks, I., 12
Lemaire, S., 358
Lemberger, L., 409
Lemire, Y., 9
Lemte, R., 159
Lenègre, J., 147
Lennard, H. L., 160
Leonards, J. R., 227, 392
Lequin, R. M., 223, 224
Lerner, A. M., 149
Lerner, R., 314
Lesch, M., 41, 42, 44-46, 56, 57
Letteri, J. M., 398
Le Valley, S. E., 36
Levan, A., 67
Levander-Lindgren, M., 148
Levey, G. S., 26
Levi, A. J., 36, 388, 413
LEVIN, A. S., 175-208; 142, 175, 177, 189, 193
LEVIN, N. W., 365-84; 376
Levin, S. R., 399
Levine, L., 226
Levine, R., 388
Levison, M. E., 86
Levitt, M., 245, 332
Levitt, M. D., 248
Levkoff, A. H., 141
Levy, G., 35-37, 387, 391, 392, 413
Levy, M. N., 369, 371, 373, 374
Levy, R. A., 138
Levy, R. I., 316-22
Levy, R. L., 141
Lewis, D., 356
Lewis, G., 404
Lewis, G. P., 388
Lewis, J. H., 12
Lewis, K. B., 63
Lewis, M. G., 202
Lewis, R. J., 394
Lewis, T. L. T., 76
Lewis, U. J., 251, 254, 267
Lewy, J. E., 228
LEWY, P. R., 365-84; 376
Leyssac, P. P., 366
L'Hermite, M., 255, 257, 258, 359
Li, C. H., 251
Li, F. P., 78
Liao, T. H., 296
Lidvall, H. F., 211
Lie, M., 379, 381
Lieberman, P., 114, 115
Liley, A. W., 167
Lilienfeld, A., 146
Lilienfeld, A. M., 76
Lillie, R. D., 167
Lim, S. D., 142
Limas, C., 141
Lin, C. C., 67, 68
Lina, P. H. C., 137
Lind, M., 34
Lind, T., 167, 168
Linden, D. A., 112
Lindenbaum, J., 105, 403
Lindquist, M , 212
Lindsay, D., 118, 119, 121
Lindsey, C. A., 303, 306
Lindsten, J., 68
Linggood, M. A., 106, 107
Linhartova, A., 121
Linman, J. W., 76

Lipmann, F., 354
Lipschultz, L. I., 86
Lipson, L. C., 20
Lipton, A., 332
Lisanti, J. A., 72
Lischner, H. W., 141
Liscio, A., 30
Little, J. B., 115, 116, 122, 126-29
Little, J. R., 370
Littledike, T., 221, 223, 224
Littlefield, J. W., 52
Livingston, J. N., 237
Livingston, R. B., 327
Ljungqvist, U., 7
Llerena, L., 265
Llerena, O., 265
Lloyd, A. V., 316
Lloyd, J. K., 316-18, 320
Lloyd-Davies, R. W., 84-86
Lobo-Antunes, J., 209-11
Lockett, M. F., 259
Lockwood, D., 237
Loeliger, E. A., 397
Loewenstein, J. E., 255
Logue, G. L., 77, 243, 347
Logue, R. B., 146
Lolekha, S., 179
Lomholt, B., 70
Lommen, E. J. P., 44, 54
Long, R. J., 30
Longnecker, R. E., 379, 381
Loranger, A. W., 209, 212
Lorente, M., 361, 362
Lortholary, P., 190
Lorusso, D. J., 394
Losowsky, M. S., 7
LoSpalluto, J. J., 19, 104
Loughran, J. M., 93
Louis, S., 397
Louro, J. M., 193
Love, A. H. G., 21, 104
Low Beer, B. V. A., 77
Lowe, C. U., 317
Lowenbraun, S., 328, 331
Lowenstein, M. S., 107
Lowman, J. T., 194
Lowry, O. H., 374
Lu, K. H., 259
Lubchenco, L. O., 172
Lubs, H., 89, 91-94
Lubs, H. A., 70
Lucas, A. R., 13
Lucas, M., 68
Lucas, Z. J., 193
Luce, J. K., 329, 332, 348, 349
Luchi, R. J., 400
Ludwig, G. D., 241
Luebbers, E., 105
Lui, P. V., 107
Lukas, D. S., 404, 405
Luke, J. L., 146
Lullin, M., 404
Lumb, G. A., 227
Lund, L., 387
Lundberg, W. B., 219
Lunde, P. K. M., 387, 398
Lundh, B., 242, 243, 246
Luomanmäki, K., 242
Luscher, E. F., 2
Lust, W. D., 20, 104
Lutterbeck, P. M., 260
Lux, S. E., 193
Lynch, S. R., 242, 243
Lynn, R., 397
Lyon, E., 146, 150
Lyon, R. P., 84, 86
Lyons, H. J., 125, 129
Lytton, B., 202

M

Maak, B., 35, 37
MacDonald, M. K., 341
Macedo, C., 209-11
MacFarlen, A., 57
MacGillwray, R., 170
MacGregor, R. R., 219, 220
MacIndoe, W. M., 260, 263
Mackenzie, M. R., 12
Macklem, P. T., 114-29
Macknight, A., 378
MacLean, L. D., 178, 181
Maclean, N., 91, 92
MacLeod, C. M., 336
MacLeod, S. M., 396
MacMahon, B., 61, 284
Madan, K., 72
Madge, G. E., 79, 80
Madison, L. L., 306, 309-11, 313
Magnani, T., 103
Maher, I. A., 1, 2
Mahler, H. R., 276
Maibach, H. I., 345-48
Maickel, R. P., 30
Maillard, J., 138
Maisel, J. C., 121
Maisels, M. J., 243
Majno, G., 19
Majunder, G. C., 267
Makowski, E. L., 168
Malaise, E., 348, 349
Malaisse, W., 233
Malamud, D., 345
Malarkey, W. B., 259
Maldonado, J. E., 76
Malherbe, C., 399
Malitz, S., 209, 412
Malkinson, F. D., 350
Malmgren, R. A., 202
Malofiejew, M., 339
Maloney, W., 75
Malvin, R. L., 368
Mammen, E., 6
Mancini, R., 149
Mandel, I., 406
Mandelbaum, I., 309
Mangelson, F. I., 317
Manitius, A., 376, 380
Mannheimer, S., 316
Manning, J., 245
Manolov, G., 68
Manolova, Y., 68
Mantegazzini, P., 212
Mantle, D. J., 91, 92
Manzke, H., 44
Marchi, P., 68
Marco, J., 303
Marcus, A. J., 1
Marcus, F., 404
Marcus, J. H., 121, 124
Marcus, S., 202
Marder, V. J., 335, 336, 338, 339
Maretic, Z., 146
Margulies, A. I., 92
Marie, J., 44
Marinetti, G. V., 239
Marini, A., 37
Marinkovich, V. A., 194
Mariz, I. K., 255, 257, 264
Mark, L. C., 389
Markham, C. H., 211
Markham, C. M , 209, 212
Markland, F. S., 336
Marks, J. F., 49, 50, 57
Marks, V., 398, 404
Marmion, B P., 191
Marmor, M. F., 193
Marsh, G. G., 209, 212
Marsh, J. B., 233
Marshall, A., 310, 313
Marshall, C. H., 26
Marshall, S. K., 326, 327
Martin, T. J , 222
Martin, W. E., 209, 212
Martin, W. R., 156
Martinez, P., 7
Martinez-Maldonado, M., 375-77, 379, 381
Marver, H., 245
Marver, H. S., 241, 244, 246-49
Marx, H. H., 370
Masi, A. T., 11
Masih, B. K., 85, 86
Massini, P., 2
Mata, L. J., 107
Matchett, O., 339
Mathe, G., 135
Matheova, E., 316
Mathews, F. L., 117, 121, 124
Mathison, D. A., 77
Matos, M., 369-73
Matschiner, J. T., 394
Matsuda, M., 5
Matsuzawa, T., 36
Matthys, H., 114
Matz, L., 35
Mauck, H. P., 64
Maude, D. L., 378, 379
Mauer, H. M., 4, 7
Maxwell, J. D., 399
Mayer, E., 327
Mayers, L. B , 242
Mayne, E. E , 8
Mayo, M., 86

McBride, J. A., 9
McCance, R. A., 168
McCann, M. L., 316
McCann, W. P., 372
McCarthy, D. S., 126
McClemont, W. F., 93
McClure, J. M. III, 86
McClure, P. D., 12
McClutcheon, E., 167
McCoy, T. A., 272
McCue, C. M., 64
MCCULLOUGH, J. L., 345-52
McDonald, J. A., 48
McDowell, F., 209, 212
McDowell, F. H., 398
McFadden, E. R. Jr., 112, 125, 129
McFarlin, D. E., 188, 191
McField, J. R., 8
McGandy, R., 316, 317
McGarry, J. M., 390
McGee, B. J., 93
McGinn, T. G., 77
McGoodwin, M. M., 319
McGuire, V. M., 91
McIlroy, M. B., 116, 119, 129
McIlwain, H., 277, 278
McIntire, M. S., 94
McIver, J., 107
McKee, D., 379
McKee, R. W., 31, 32
McKeel, D. W., 251
McKeown, T., 61
McKerrow, C. B., 125
McKusick, V. A., 61
McLachlan, D. G., 92
McLean, E. P., 179, 202
McLean, F., 172
McLean, K. H., 121
McLean, R. H., 62
McLeod, P. J., 396
McMahon, F. G., 396
McNamara, D. G., 61
McNamara, H., 317
McNamara, P. M., 318
McNicol, G. P., 10, 13, 338
McPhedran, P., 77, 78, 395
McQuaid, M., 91
Mead, J., 114-19, 121-24, 126-29, 168
Meade, J. C., 48
Meagher, D. M., 6
Meeker, B. E., 332, 350
Meier, M. J., 209, 212
Meites, J., 259, 265
Melichar, V., 316
Melick, R. A., 222
Mellemgaard, K., 117
Mellman, W. J., 91
Mellow, M., 403
Melmon, K. M., 401
Melnick, I., 83
Melson, G. L., 224, 226
Melville, M. M., 93
Mendez, J., 316
Menkes, H., 118, 119, 121
Menolascino, F. J., 94
Menzer-Benaron, D., 90, 91
Mercke, C., 242, 243, 246
Meriel, P., 370
Merker, H. J., 245
Merlino, N., 212
Merlis, S., 211
Merrick, S., 70
Merrill, J. P., 341
Merskey, C., 339
Messeloff, C. R., 210
Metcalfe, S., 165
Metcoff, J., 366
Meuwissen, H. J., 136, 140, 193
Meyer, T. C., 146
Meyler, L., 78
Michael, A., 397
Michel, H., 12
Michel, M., 43, 44
Michener, W. M., 41, 57
Middlemas, R. O. III, 193
Midgley, A. R., 255, 257, 258
Midgley, A. R. Jr., 295-97
Mielki, C. H. Jr., 1, 2
Migeon, B. R., 49, 50
Mihara, G., 83, 84
Mijeon, C. J., 35
Miles, A. A., 290
Miles, E. M., 290
Miles, P. A., 168
Milic-Emili, J., 117, 118, 126
Milla, E., 366
Millar, J. H. D., 8
Millar, M. A., 83, 84
Millen, R. S., 316
Miller, D. A., 68
Miller, E. R., 86
Miller, E. V., 167
Miller, G. M., 86
Miller, J., 47, 56
Miller, J. F. A. P., 196
Miller, L., 321
Miller, M. E., 136, 192
Miller, M. W., 128
Miller, O. J., 67, 68, 70, 91
Miller, R. G., 137
Miller, R. W., 80
Miller, S. A., 275
Miller, W. F., 129
Milligan, C. A., 317
Mills, I. H., 372
Milstone, L. M., 179
Mindham, R. H. S., 209
Miranda, Q. R., 149
Miras, C. J., 9
Mireault, N. G., 42
Mirkin, B. L., 34
Mirsky, I. A., 309, 314
Mischel, W., 97
Misenheimer, H. R , 167
Mitchell, C. A., 124
Mitchell, G. F., 196
Mitchell, J. R., 3, 411
Mitchell, J. R. A., 1, 3, 5, 6, 8, 10, 11, 13
Mitchell, R. G., 172
Mitchell, R. S , 121
Mitchell, S. C., 61
Mitnick, M. A., 354, 355
Mitoma, C., 36
Mittelman, A., 284
Mittenthal, S., 90, 91, 97
Miyai, K., 361
Mobbs, G. A., 85
Modan, B., 76
Modest, E. J., 67
Moede, A. L., 242, 243
Mohr, J., 70
Moir, D. C., 412
Molinas, F., 8
Moller, G., 202
Moller, J. H., 62
Mönch, H., 6
Mond, N. C., 86
Money, J., 89-93, 97
Monif, G. R. G., 146
Montgomerie, J. Z., 84
Montgomery, D., 2
Monto, A. S., 83
Moon, H. W., 106, 107
Mooney, J. J., 179
Moore, D. E., 70
Moore, E. W., 105
Moore, G. E., 203
Moore, M. R., 325, 327, 331
Moore, T., 83
Moore, W J., 21
Moore, W. M. O., 168
Moores, E. C., 63
Moorhead, D. T , 212
MOOS, R. H., 89-102; 93, 96, 97
Morawski, S. G., 21
Morel, A. G., 296
Morgan, A. P., 369-73
Morgan, C. R., 309
Morgan, E. J., 125
Morgan, F. J., 295, 297
Morgan, F. M., 103
Morgan, L. L., 53
Morgan, T. E., 128
Moritz, M., 105
Morris, E. D., 168
Morris, H. H. B., 167
Morris, J. J. Jr., 400
Morris, T. A. Jr., 90, 91
Morrow, A. G., 64
Morselli, P. L., 398
Mortimer, J. G , 316
Mortimer, W. C., 85
Mortimore, G. E., 309-11
Morton, D. L., 202
Moser, W., 191
Mosesson, M. W., 336
Mosher, M., 389, 413
Mosko, M. M., 176, 177
Mosley, W. H , 105

Mosovich, L. L., 317
Motoyama, E. K., 120, 126, 168
Motulsky, A. G., 51, 63, 319
Mourek, J., 278
Moxley, J. H. III, 331
Moylan, J. A., 303, 306
Muckle, T. J., 13
Mudge, G. H., 376
Mueller-Eckhardt, C., 6
Mueller-Hartburg, W., 316
Muir, A., 118, 119, 121
Muir, D. C. F., 117, 122
Muittari, A., 130
Müller, M., 265
Müller, W., 278
Müller, W. A., 303-6
Muller-Eberhard, V., 168
Muna, N. M., 202
Munck, O., 366, 367
Muñoz-Armas, S., 63
Munson, P. L., 218, 226
Murat, L. G., 340
Murnaghan, G. F., 84
MURPHY, D. L., 209-16; 209-14
Murphy, M. L., 404
Murphy, P. J., 168
Murphy, S., 4
Murray, J., 168
Murtaugh, P. A., 339
Musshoff, K., 325, 329
Mustard, J. F., 1, 6
Musto, D., 154
Myat, G., 4
Myllylä, G., 5
Myres, R. P., 255

N

Nabarro, J. D., 361
Nabarro, J. D. N., 314
Nachman, R. L., 4
Nadal, J. A., 111, 124, 127
Nadler, C. W., 276, 278
Nadler, S. H., 203
Naeye, R. L., 170
Nagasawa, H., 265
Nail, B., 259
Naiman, J. L., 7
Nair, R., 355
Nakama, M., 212
Nakamura, H., 105
Nakamura, J., 169
Nakatomi, M., 105
Nalin, D. R., 105
Narahara, H. T., 233
Nasca, P. C., 414
Nash, F. D., 366-69, 376, 379
Natarajan, N., 76
Nathan, C. F., 179
Nathan, D. G., 78, 196
Nathan, R., 201
Nathenson, G., 413
Neal, A. J., 79
Nechay, B. R., 379-81
Needleman, P., 374
Neemeh, J. A., 12
Negus, D., 6
NEILL, C. A., 61-66; 61, 62, 64
Neith, H., 369, 370, 372
Nelson, G. H., 169
Nelson, J. A., 379-81
Nelson, L. G., 56
Nelson, N. C., 310, 311, 313
Nelson, R. M., 294
Nemechek, C., 36
Nencioni, T., 316
Nepszy, S. J., 120
Netter, K. J., 32
Neumann, J., 315
Neville, D. M., 234, 235, 237
Newall, J., 332
Newby, J. P., 120
Newcombe, D. S., 46, 49, 57
Newerly, K., 233
Newman, D. R., 76
Newman, G. B., 170, 171, 314
Newsholme, E. A., 375
Newsom Davis, J., 111
Newsome, J. F., 332
Nezelof, C., 190
Niall, H., 254
Niall, H. D., 218
Nicholls, D. G., 11
Nichols, J., 63
Nicholson, D. C., 241
Nicholson, W. M., 328
Nickel, E., 86
Nickson, J. J., 326
Nicoll, C. S., 255, 267
Nielsen, J., 91, 92
Nielsen, N. C., 276
Nies, A. S., 389
Nieschlag, E., 299
Nieth, H., 370
Niewiarowski, S., 6, 338, 340, 341
Niewoehner, D. E., 112
Nigrin, G., 37
Niilo, L., 107
Nijessen, J. G., 3
Niki, H., 249
Nilsson, I. M., 7, 11, 12, 341
Nilsson, L., 11
Niosi, P., 138
Nishiitsutsuji-Uwo, J. M., 370-73
Nissen, N. I., 328, 329
Niswender, G. D., 296, 297
Nitowsky, H. M., 35
Nitzan, M., 316
Niwinski, R. C., 48
Noall, M. W., 237
Noel, G., 252, 256, 257, 259
Noftle, K. A., 19, 104
Nokin, J., 255, 257, 258
Nolan, M. F., 369, 370, 373
Nolan, T. B., 146
Noll, R. M., 378, 379
Noonan, J. A., 63
Noone, P., 387
Nora, J. J., 61-63
Nordbring, F., 148
Norden, C. W., 84
Nordenson, N. G., 77
Nordgren, B., 148
Nordman, R., 316
Nordöy, A., 8, 9
Noren, G. R., 149
Norris, H. T., 19
Norris, J. L., 381
North, J. D. K., 84
Northrup, R. S., 105
Norton, R. A., 77
Novak, M., 316
Nussenzweig, V., 336
Nutter, S., 392
NYHAN, W. L., 41-60; 41, 42, 44-57
Nyswander, M. E., 154, 155, 157

O

Oates, J., 397
Oates, J. A., 389, 410, 411
O'Brien, C. P., 209-11
O'Brien, J., 337, 341
O'Brien, J. R., 1, 2, 4, 6, 10
O'Brien, R. A., 13
Öckerman, P. A., 7
Odakura, T., 9, 10
Odar-Cederlöf, I., 398
ODELL, G. B., 165-74
Odell, W. D., 356, 357
Odland, L. M., 317
O'Donoghue, L. E. J., 34
Odoroff, C. L., 396
Oettgen, H., 203
Oettgen, H. F., 179, 202
Offenkrantz, F. M., 317
Ogawa, H., 105
Ogg, C. S., 77
Ohata, M., 222
Ohneda, A., 304
Oka, T., 237
Okeson, G. C., 126
Okonkwo, P., 5
Olafsson, S., 123
Old, L. J., 179, 202
Oldham, S. B., 219-22
Olesen, O. V., 398
Oliner, H. L., 282
Olinger, C., 399
Oliver, D. R., 114, 119
Olsen, C. R., 116, 119, 129

Olsen, D. E., 114
O'Malley, K., 390
O'Neal, R. M., 121
Ono, M., 237
Onoshi, T., 332
Opie, L. H., 114, 124
Oram, S., 63
O'Reilly, R. A., 393
Orha, I., 122, 127
Orimo, H., 222
Oriol, A., 125
O'Riordan, J. L. H., 218, 220, 226-28
O'Riordan, M. L., 68
Orloff, J., 366, 373
Orme, M., 397
Ormston, B. J., 358, 360, 361
Orndahl, G., 146
Orrenius, S., 32-34
Orsi, E. V., 149
Osborn, N., 338
Oseasohn, R. O., 105
Osgood, E. E., 77
Oski, F. A., 193
Osserman, E. F., 78
Ostle, R. J., 317
Ott, J. E., 13
Overton, D. A., 212
Owen, C. A., 12
Owen, C. A. Jr., 2, 3, 5
Owen, O. E., 273, 275, 276, 369-73
Owens, P. R., 114
Owren, P. A., 335
Oxley, A., 168

P

Packham, M. A., 1
Padeh, B., 283
Page, A. R., 196
Page, J. G., 390
Page, M. A., 273, 275-78
Pagtakhan, R. D., 34
Pak, C. Y., 229
Palade, G. E., 31
Palmer, R. F., 379, 380
Palos, B., 327, 328
Pantelakis, S. N., 316
Pantuck, E. J., 36, 390
Papaioannou, R., 47
Papayoanou, M. F., 3, 7
Papkoff, H., 296
Pappenheimer, A. M., 203
Pappenheimer, J. R., 368, 374
Pardue, M. L., 70
Pare, C. M. B., 92
Parekh, S. J., 4
PARK, B. H., 135-44; 137, 138
Parker, I., 117
Parkin, D. M., 135
Parkin, F. M., 168
Parkinson, D. K., 20, 24
Parks, G. H., 77
Parks, R. E. Jr., 77
Parlow, A. F., 296
Parmley, T., 167
Parrow, A., 148
Parson, W., 224
Parsons, C. G., 61
Parsons, V., 399
Partington, M. W., 42
Pasqualini, R. Q., 92, 93
Passananti, G. T., 390 409
Passonneau, J. V., 374
Pasteels, J. L., 252, 253
Patch, V. D., 162
Paterson, J. W., 402
Patil, S. R., 70
Patterson, D. F., 62
Patterson, J. F., 77
Pattijn, J., 114, 115, 123
Pattison, J. R., 387
Paul, W., 380
Paulsen, C. A., 295
Paulson, G. W., 211
Paupe, J., 316
Pavilanis, V., 316
Pazery, L. M., 212, 213
Peake, G. T., 251, 256
Pearce, J. M., 149
Pearce, M. L., 405
Pearce, W., 316, 320
Pearson, H. A., 193
Pearson, H. E., 146
Pearson, J., 12
Pearson, J. W., 168
Pearson, O. H., 265
Pearson, P. L., 68, 72
Peberdy, M., 11, 12
Peddle, L. J., 167
Pedley, T. J., 114, 115, 117
Pegrum, G. D., 8
Pehling, G., 375
Pek, S., 303, 306
Pelissier, N. A., 387
Pelkonen, O., 32, 33, 37
Pello, F., 284
Pelmont, J., 336
Penn, I., 79
Penneys, N. S., 348
Penny, R., 12
Pennybacker, W., 76
Penrose, L. S., 94
Penttinen, K., 5
Peralta, H., 149
Percival, A., 86
Percy, K., 30
Pereira, J. P. M., 347
Perel, J. M., 389, 412
Perey, D. Y., 135
Perkins, H. A., 12
Perkins, J., 77
Perleffi, L., 37
Permutt, S., 115, 116, 123, 128
Perrier, D., 149
Perry, H. M. Jr., 407
Perry, H. O., 350
Perry, S., 327
Persson, B., 275, 277, 278, 316, 317
Persson, U., 277, 278
Pesek, J., 49
Pessin, V., 317
Peter, H. M., 374, 376
Peters, J. M., 111, 112, 127
Peters, M. V., 329
Petersdorf, R. G , 83
Peterson, C. A., 294
Peterson, J., 2
Peterson, R. D. A., 80, 191, 135
Petkov, G., 7
Petrie, J. J. B., 341
Petryka, Z.; 241
Petty, T. L., 121, 128
Philbin, P. E., 414
Philip, A. E., 93
Phillips, G. B., 316
Phillips, P. E., 7, 8
Phillips, R. A., 21, 103, 104, 137
Phillipson, O. T., 11
Pick, E., 179
Picken, J. J., 112
Piel, C. F., 228
Pierce, A. K., 129
Pierce, G. E., 202
Pierce, N. F., 19, 20, 26, 105, 106
Pierce, J. C., 79, 80
Pierce, J. G., 296, 297
Piermattel, D. L., 6
Pierre, R. V., 77
Pierson, P. S., 162
Pike, B. L., 286
Pikkarainen, P., 33
Pillow, R. P., 135
Pinckard, R. N., 388
Pinkhas, J., 12, 283
Pinsky, L., 42
Pinto, D. J., 6
Piomelli, S., 48
Pipat, C., 27
Piscatelli, R. L., 393
Pitkin, R. M., 168
Pittman, J., 353, 357, 358, 360, 361
Piyaratn, P., 19
Place, R. E. G., 125
Pletka, P., 228
Plotkin, G. R., 19, 20, 104
Poirier, G., 358
Poláček, K., 168
Polasek, J., 6
Polani, P. E., 61-63, 89, 90, 91
Polis, B. D., 276, 278
Polishuk, W. Z., 316
Poller, L., 4, 11
Pollock, P. G., 392
Pollock, T., 167
Pomales, R., 57
Pomeranze, J., 317

Pomeroy, J. R., 390
Pomp, H., 32
Poole, E. W., 93
Poppius, H., 130
Porter, D., 346, 347
Porter, D. I., 350
Porter, H. M., 186
Porter, J., 356
Posey, V. A., 379, 380
Poskanzer, D., 414
Post, R., 5
Post, R. L., 378
Poth, J. L., 76
POTOLSKY, A., 75-82
Potter, D., 380
Potter, E. V., 341
Potter, H. A., 379
Potter, W. A., 123
Potts, J. T. Jr., 217-20, 222, 223, 225, 227
Poulter, C., 327
Powell, D., 226
Powell, D. W., 22
Pozsonyi, J., 94
Pratt, R. T. C., 409
Preece, J. M., 398
Prentice, C. R. M., 338
Prescott, L. F., 385
Price, W. H., 93, 98
Price, Z. H., 31, 32
Pride, N. B., 115, 116, 119, 123, 128, 129
Priest, C. M., 4, 11
Pritchard, M., 92
Proctor, D. F., 113
Prosnitz, L. R., 325, 326
Proulx, R. A., 393
Proverbio, F., 378, 379
Pruitt, R. D., 148
Pryor, J. S., 260
Prystowsky, H., 168
Przegalinski, E., 212
Puck, T. T., 95
Puletti, F., 209, 212
Pull, I., 277, 278
Purcell, I. M., 13
Purves, H. D., 252

Q

Quastel, J. H., 277, 278
Quastler, H., 345
Quie, P. G., 193
Quigley, J. P., 24
QUINTANILLA, A., 365-84; 376

R

Raab, W. P., 387
Rabiner, S. F., 8
Rado, E., 405
Rafelson, M. Jr., 4
Rafstedt, S., 315-17, 321
Rahman, M., 105
Raiha, N. C. R., 33
Rainer, W. G., 120
RAISFELD, I. H., 385-418; 385, 389, 411
Raisz, L. G., 396
Raitt, J. W., 77
Ramanan, C. V., 283
Ramos, G., 369-73
Rampone, J. F., 294
Ramsey, H. E., 325, 326
Randall, H. M., 371
Rane, A., 32-34, 398
Rangno, R. E., 402
Rantz, L. A., 83
Rapaport, F. F., 142
Rapaport, S. I., 1, 2
Rapoport, B., 261
Rappaport, R., 44
Rasco, M. A., 296
Rasia, M. L., 378
Raskin, N. H., 273
Rasmussen, H., 218
Rasmussen, K., 405
Ratcliff, R. A. W., 93
Rathbone, R. L., 8
Rathey, U., 211
Rathnam, P., 297
Ratnoff, O. D., 336
Rauwerda, P. E., 117
Ray, T. K., 239
Rayford, P. L., 297
Raymunt, J., 316
Read, J., 124
Ream, V. J., 7, 338
Reaven, G. M., 309, 314
Redding, T., 355, 356
Redeker, A. G., 246
Redwood, D., 146
Reed, W. B., 42
Rees, R. J. W., 179
Reeves, R. B., 368, 374
Refetoff, S., 261, 357
Refsum, H. E., 366, 367
Regnault, F., 8
Regoeczi, E., 6
Reich, S., 404
Reichert, L. E. Jr., 296-99
Reichlin, S., 353-56
Reidenberg, M. M., 398, 414
Reilly, C. J., 332
Reimers, H. J., 9
Reinicke, C., 35, 37
Reis, D. J., 212
Reisman, L. E., 94
REISS, E., 217-32; 220, 221, 223-29
Reitz, R. E., 226
Remmer, H., 245
Remold, H. G., 177, 179
Renaud, S., 9
Rennie, D. W., 366-38, 374
Revillard, J. P., 191, 192
Reyes, H., 36, 388
Reynolds, J. C., 227
Rhode, J. E., 20, 21, 23, 24, 26
Rhodin, J., 373
Rich, A., 219
Rich, R. R., 193
Richards, B. W., 93
Richardson, C. E., 91, 93
Richardson, J. A., 414
Richardson, S. H., 19, 25, 104
Riches, H. R. C., 193
Richet, G., 21
Richmond, S., 93
Rickard, S., 2
Rickles, F. R., 396
Riddle, J. M , 10
Ridler, M. A. C., 70, 91
Riedler, G. F., 5
Riegelman, S., 387
Rieser, P., 233
Rifkin, P. L., 3
Riggs, B. L., 227
Rigollot, C., 6
Rigsby, W. C., 167
Riklan, M., 209, 212
Riley, R. L., 115, 116, 123, 128
Rimoin, D. L., 251
Rind, H., 34
Ringel, S. P., 211
Ritschard, J., 9
Rivier, J., 353, 354, 358
Rizzo, M., 398
Robach, J., 92
Robbins, J. B., 299
Roberts, A. P., 85
Robertson, D. M., 44
Robertson, J. H., 283
Robertson, L. W., 64
Robertson, M. R., 79
Robertson, P. C., 126
Robins, E., 412
Robinson, A., 95, 97, 320
Robinson, D. E., 170
Robinson, J. A., 68
Robinson, J W. L., 378, 379
Robinson, S. H., 245
Robinson, W. A., 286
Robison, G. A., 26
Robson, J. S., 341
Robyn, C., 255, 257, 258
Rocklin, R. E., 193
Rockoff, S. D., 64
Rodbard, D., 295
Rodbell, M., 234
Rodkey, F. L., 244-48
Rodman, J. S., 219
Rodman, N. F., 5
Rödset, J. M., 8, 9
Roe, D. A., 390
Rogers, A. B., 2
Rogers, J., 2, 4, 5
Rogers, L. A., 31
Rogers, R. M., 128
Rogner, G., 35, 37
Rohde, J. E., 104
Rohwedder, H. J., 35
Roman, I. C., 72
Ronan, R., 218
Roof, B. S., 223, 224, 226-28

Rook, A., 68
Roopnarinesingh, S., 168
Rose, R., 11
Rosecrans, C. J., 94
Rosemberg, E., 295
Rosen, F. S., 62, 141, 186, 188
Rosen, O. M., 107
Rosen, S. M., 107
Rosen, S. W., 299
Rosenberg, C. M., 162
Rosenberg, I. H., 24, 398
Rosenberg, J. C., 6
Rosenberg, M. M., 44, 45
Rosenberg, S. A., 77, 325, 327, 331
Rosenbloom, F. M., 41, 45-48, 50, 54-56
Rosenfield, R. E., 168
Rosengren, B., 7
Rosenkranz, W., 91
Rosenthal, H. S., 160
Ross, B. D., 370-73
Ross, E. M., 7
ROSS, G. T., 295-302; 295-99
Ross, J. C., 112, 129
Ross, J. J., 64
Ross, W. R. D., 126
Rosse, W. F., 247
Rossi, E., 188
Roth, J., 234, 235, 237, 251, 299
Roth, L., 368
Roth, S. I., 225
Rothberg, S., 346, 347
Roux, J. F., 169
Rowland, M., 387, 401
Rowley, J. D., 68
Roxe, D. M., 372, 373
Royer, J. M., 44
Royer, P., 44
Rubin, C. S., 48
Rubin, P., 327
Rubini, J. R., 345
Rubio, F., 282
Ruddle, F., 68
Ruddle, F. H., 70
Ruddle, N. H., 179
Ruff, F., 126
Rundles, R. W., 77
Russ, E. M., 316
Russell, S. H., 165
Rutenberg, H. L., 316
Ruthven, C. R. J., 168
Ryan, J. R., 396
Ryan, R. J., 295, 297
Ryden, G., 167
Ryder, J. A., 310, 311, 313
Rytömaa, T., 286

S

Saarni, M. I., 78
Sabata, V., 316
Sabbath, J. C., 90, 91
Sabesin, S. M., 24
Sack, R. B., 19, 103-6
Sacks, O. W., 210, 212
Saenger, E. L., 76
Sahud, M. A., 7, 392
Saimyoji, H., 368
Sainani, G. S., 146, 149
Saita, G., 79
Saito, S., 360, 361
Sakai, T., 380
Sakamoto, A., 407
Sakamoto, Y., 89, 90
Sakasaki, R., 105
Sakiz, E., 357
Sakoda, M., 357, 358, 361
Salmon, S. E., 140
Salomon, L., 92
Saltzstein, E. C., 138
Salzberg, R., 35
Salzman, E. W., 13
Salzman, J. R., 325
Salzmann, J., 49, 50
Samols, E., 310, 311, 313
Samy, T. S. A., 296
Sanbar, S. S., 8
Sandberg, A. A., 76, 235, 327
Sanders, B. C., 294
Sandler, M., 213
Sanford, J. P., 85
Sano, T., 4, 9, 10
Santeusanio, F., 303, 304, 306
Santos, G. W., 140
Saphir, O., 145, 147
Saraya, A. K., 12
Sarji, K. E., 5
Sarto, G., 51
Sass, J. K., 44
Satoh, M., 212
Satterthwaite, H. S., 277
Sauceda, G., 374
Sauer, L. A., 376
Saunders, G. F., 72
Savits, B. S., 316
Savoie, L., 406
Savola, J., 148
Saxena, B. B., 297
Scadding, J. G., 112
Scaglione, P. R., 79
Schabel, F. M., 350
Schacter, B. A., 245, 248
Schadlow, A., 358
Schafer, D. E., 20, 106
Schaffner, F., 389
Schaller, J., 192
Schally, A., 353, 355, 356, 358, 360, 361
Schatz, I. J., 10
Schear, M. J., 210
Scheckel, C. L., 212, 213
Scheinberg, I. H., 396
Schidlovsky, G., 202
Schiffer, L. M., 210
Schilder, D. P., 122, 123
Schimke, R. N., 193
Schimke, R. T., 277
Schiodt, T., 202
Schlaff, S., 299
Schlein, E. M., 304
Schlom, I., 265
Schmaelzle, J. F., 86
Schmahl, F. W., 9
Schmid, R., 241, 245-49
Schmidt, C. F., 276, 278
Schmidt, U., 379, 381
Schnedl, W., 70
Schneider, H., 103
Schneiderman, N., 53
Schnoor, M., 32
Schoenfeld, M., 316, 320
Schoenfeld, R. I., 213
Schollmeyer, P., 369, 370, 372
Schopman, W., 223, 224
Schreiber, E C., 401
Schreiber, W., 146, 148
Schretlen, E. D. A. M., 44, 45
Schrier, S. L , 76
Schroeder, J., 72
Schroter, R., 118, 119, 121
Schroter, R. C., 114, 115, 117
Schrott, H. G., 319
Schuchkit, M., 412
Schulkind, M. L., 177, 193
Schulte, F. J., 172
Schuster, C. R., 163
Schuurs, A. H. W. M., 296
Schwartz, A., 379, 381
Schwartz, A. G., 53
Schwartz, C. J., 8
Schwartz, R., 316
Schwartz, R. S., 77, 282
Schwartz, W. F., 210
Schwarz, G. A., 209-11
Schwarz, G. S., 171
Schwarz, M. I., 327
Schweisguth, O., 61
Schweppe, H. I., 121
Scott, K. E., 172
Scott, R. C., 51
Scrimshaw, N. S., 103, 316, 317
Scriver, C., 228
Seabright, M., 70, 72
Sealy, R., 329
Seaman, A., 77
Seavey, B. K., 251, 254
Sedgwick, R. P., 191
Seegmiller, J. E., 41, 45-48, 50-52, 54-56
Seeler, R., 4
Seely, J. E., 112, 122, 126, 127
Seibel, M. J., 357
Seip, M., 193
Sejersted, O. M., 379, 381
Sekiguchi, M., 145
Selawry, O. S., 332
Self, T. W., 188
Seligmann, M., 336
Selleck, B. H., 371

Sellei, C., 284
Sellers, E. M., 388, 393, 395, 396
Selye, H., 406
Selzer, G., 329
SENAY, E. C., 153-64; 163
Senn, M., 317
Senyi, A. F., 6
Serebro, H. A., 26, 105
Sereni, F., 37
Sergovich, F. R., 91
Serpick, A. A., 325, 326, 328, 331, 332
Serra, M. T., 408
Settergren, G., 275, 278
Sevdalian, D. A., 276
Shacks, S. J., 179
Shaffer, J. W., 90, 91
Shah, S. A., 95-97
Shahidi, N. T., 389
Shainoff, J. R., 336
Shaller, J., 188
Shand, D. G., 402, 403, 411
Shannon, D. C., 186
Shanske, A., 389, 413
Shapiro, S., 393
Shapiro, S. L., 49, 57
Sharkey, J., 210
Sharman, D. F., 212
Sharp, F., 167
SHARP, G. W. G., 19-28; 20, 22-24, 26, 104
Sharpstone, P., 77
Shaver, E. L., 91
Shaw, S., 8
Shaw, M. W., 70, 80
Sheiffer, S., 37
Shellam, G. R., 196
Shelly, H. J., 31
Shepard, T. H., 295
Sheppard, G. L., 57
Sheppard, G. L. Jr., 49
Sherins, R. J., 331
Sherman, F. G., 345
Sherman, L. A., 336
Sherry, S., 335, 336, 338, 339
Sherwood, L. M., 218-20, 223, 225-27, 251
Shimamoto, T., 10
Shimkin, P., 273
Shipe, D., 94
Shiraishi, Y., 70
Shirakawa, S., 348, 349
Shirkey, H. C., 392
Shirkey, H. S., 196
Shiu, R., 255
Shiurba, R., 140
Shoeman, D. W., 387, 398, 405
Shliton, L., 399
Shoobridge, S. M., 1
Shore, E., 107
Short, C. R., 30, 31
Shullenberger, C. C., 283, 327, 329
Shulman, N. R., 1, 6, 193, 335, 336, 338, 339
Shuster, S., 346, 347
Siddons, H., 146
Siegel, N. H., 7
Siekevitz, P., 31
Siersbaek-Nielsen, K., 397
Sievers, J., 63
Siew, S., 147
Sigell, L. T., 393
Silen, W., 19, 20
Siltzbach, L. E., 198
Silver, M. J., 7
Silver, R. T., 331
Silverman, A. H., 146
Silvers, A., 309, 314
Silvers, D. N., 51
Silvers, G. W., 121
Simes, E. L., 72
Simon, A. B., 229
Simon, C., 35
Simpson, C. L., 76
Sinakos, Z., 12
Singh, R. N. P., 251, 254
Sinha, Y. N., 254, 267
Sinks, L. F., 332
Siomopoulos, V., 412
Sipova, I., 92
Sircar, B., 20, 104
Sise, H., 5
Siskind, G. W., 6
Sissman, N. J., 64
Sixma, J. J., 3
Sizemore, G. W., 220-22
Sjögren, A., 3, 10
Sjogren, H. O., 202
Sjoqvist, F., 32-34, 390, 398, 408, 409
Sjöstrand, T., 241
Skerman, F. J., 106
Skipper, H. E., 350
Skou, J. C., 378
Skovsted, L., 397
Skoza, L., 1, 2
Skulan, T. W., 368
Slack, J., 319
Slack, N. H., 332
Slack, W. W., 6
Slater, E., 89, 96, 97
Slatopolsky, E., 227
Slichter, S. J., 1
Slodki, S. J., 146, 149
Sloman, G., 64
Slone, D., 393
Sloviter, H. A., 272, 273
Smart, C., 202
Smisson, C., 168
Smit, C. G. S., 78
Smith, A. L., 277
Smith, B. B., 163
Smith, D. W., 92, 93, 99
Smith, F. G., 229
Smith, G. F., 13, 91, 94
Smith, H. W., 106, 107
Smith, J. W., 85
Smith, L. H., 227
Smith, M. E., 396
Smith, M. M., 124
Smith, N., 106
Smith, P. G., 76
Smith, P. H., 224
Smith, T. T., 178, 181
Smith, T. W., 403, 405, 506
Smith, W. G., 145, 146, 148
Smithers, D. W., 80
Smyth, M. B., 171
Sneddon, J. M., 12
Snell, M. M., 389
Snyder, P., 358-62
Snyder, P. J., 261
Snyder, S. H., 214
Sogame, S., 105
Sohal, R. S., 145, 149
Sohar, E., 316
SOKAL, J. E., 281-88; 76, 284-86, 327
SOKOLOFF, L., 271-80; 271-73, 275, 277, 278, 355
Soloff, L. A., 316
Solomon, H. M., 385, 397, 404
Solomons, C. C , 13
Solum, N. O., 4
Somerville, R. J., 61
Sommerville, J., 63
Sones, M., 199
Soothill, J. F., 138
Soppitt, G. D., 11
Sørensen, A., 92
South, M. A., 193
Southam, C. M., 202
Southcombe, C., 167
Southgate, J., 13
Soyka, L. F., 30, 33
Spaet, T. H., 3, 12
Spagnuolo, M., 3
Sparkes, R., 51
Sparkes, R. S., 93
Sparrow, A. W., 7
Spaulding, S., 362
Spector, S., 159
Spellacy, W. N., 167, 169
Spencer, F. C., 64
Spencer, R., 126
Sperber, M. A., 92
Sperry, W. M., 316, 317
Spicuzza, T. J., 64
Spiegelman, S., 265
Spielvogel, A. R., 4
Spierto, F. W., 220
Spirt, N., 404
SPITLER, L. E., 175-208; 142, 175, 177-79, 188, 193, 194
Spitzer, J. J., 275, 276, 278, 369, 370
Spivey, G. H., 105
Sprinz, H., 19, 25, 103
Spronle, W. B., 169
Sroczynski, J., 5
Stachurska, J., 6, 338
Stadie, W. C., 233
St. Geme, J. W. Jr., 149
Staley, H. L., 316

Stambler, M., 92
Stamey, T. A., 83-85
Stanbury, S. W., 227
Stănescu, D. C., 114, 115, 123
Stanton, R., 7
Starkie, C. M., 317
Starkweather, D. K., 388
Starzl, T. E., 79
Staub, J., 360
Staub, N. C., 116, 117, 119, 120, 129
Steel, C. M., 68
Steel, G. G., 350
Steele, R., 392
Steele, R. W., 141
Steenberg, R. W., 19
Steffel, H. C., 228
Stein, E., 370
Steinberg, A. G., 191
Steinberg, D., 26
Steinberg, M. H., 76
Steiner, A., 358
Steiner, D. F., 219
Steiner, M., 4
Steiner, P., 316, 320
Steinman, R., 6
Stenliep, I., 296
Stenson, R. E., 401
Sterling, G. M., 127
Sterling, R. E., 246
Stern, G. M., 210
Stern, L., 37
Stern, M. P., 309, 314
Sternberg, J., 316
Stetten, D. Jr., 309-11
Stevens, A. E., 116, 119, 129
Stevens, P. J., 147
Stevenson, I. H., 390
Stevenson, R. E., 62
Stewart, A., 76
Stewart, A. D., 169
Stewart, D. A., 399
Stewart, G. J., 6
Stewart, J. R., 327
Stewart, J. W., 135
Stewart, R. D., 249
Stewart, T. H. M., 202
Stickel, D. L., 138
Stiehm, C. R., 341
Stipes, A., 98, 99
Stites, D. P., 142, 175, 177, 178, 181, 190
Stjernswärd, J., 202
Stocker, F., 188
Stockert, J. C., 72
Stoker, G. L., 129
Stoll, B. A., 266
Stolle, L. C., 21
Stone, J. D., 103
Storb, R., 139, 140
Stormorken, H., 10
Storstein, L., 405
Stouffer, J. E., 237
Stran, H. M., 166
Strang, R. H., 61
Straub, P. W., 5, 340
Strauss, J., 368
Strean, G. J., 316
Street, D. R. K., 93
Streicher, H., 70
Streiff, R. R., 35
Streuli, F., 8, 9
Strickland, W., 310, 313
Strickler, J. C., 368, 375
Strober, W., 188, 191
Strong, D. H., 107
Strong, J. A., 92
Strong, R. R., 4
Stuart, R. K., 7
Studd, J. W., 317
Stumme, E., 252
Sturgis, S. H., 90, 91
Stutman, O., 138, 141
STUTZMAN, L., 325-34; 326, 328-30, 332
Stutzman, M., 326
Subak-Sharpe, J. H., 52
Subong, A., 105
Sudduth, H. C., 276, 277
Sudlow, M. F., 114, 115, 117
Suhara, K., 273
Suki, W. N., 375-77, 379, 381
Sullivan, I., 130, 414
Sultan, Y., 12
Sumner, A. T., 68, 70 72
Sun, S. C., 145, 149
Sunden, B., 169, 171
Sussman, K. E., 310, 313, 314
Sutherland, E. W., 26
Sutherland, J., 325, 326
Suttie, J. W., 394
Sutton, G. C., 145
Suzuki, F., 237
Svahn-Tapper, G., 327
Svejgaard, A., 137
Swahn, B., 316, 317, 321
Swaminathan, N., 297
Swan, H., 6
Swanson, D., 98, 99
Swanson, S., 62
Sweeney, M. J., 316, 317
Sweet, A. Y., 169
Sweet, B., 336
Sweetman, L., 46-49, 54-57
Swidler, G., 385
Sylvester, P. E., 398
Symons, C., 4
Szanto, S. F., 5
Sze, Y. L., 218
Szentivanyi, A., 127, 129
Szepesi, B., 374
Szirtes, M., 8

T

Taggart, J. V., 376
Takagi, H., 212
Takakura, I., 105
Takano, J , 332
Takeda, Y., 237
Takeuchi, A., 107
Takeuchi, K., 9, 10
Takishima, T., 118, 119, 121
Takki, S., 402
Talkington, L. W , 98
Tamatsu, T., 123
Tan, E. M., 407
Tanagho, E. A., 84, 86
Tangun, Y., 12
Tanigawa, Y., 89, 90
Tannock, I., 348, 349
Tashjian, A. H., 262
Tashjian, A. H. Jr., 222, 224, 226
Tatter, D., 146
Taussig, H. B., 64
Taves, D. R., 228
Taylor, A. I., 91
Taylor, A. J. W., 92
Taylor, G. E., 316
Taylor, H. F., 121, 129
Taylor, J. R., 347
Taylor, J. S., 84
Taylor, W. R., 373
Teague, P., 141
Teberg, A. J., 56
Teisseyre, E., 337
Telfer, M. A., 47, 52, 91, 93
Tell, G. P. E., 237, 238
Temple, R., 357
Tenhunen, R. T., 241, 244, 246-49
Tenstam, J., 93
Teoh, E. S., 167
Terasaki, P. I., 136
Terry, L. N. Jr., 332
Tervila, L., 316
Thaler, M. M., 245, 247, 277, 278, 413
Thaysen, J. H., 366, 367
Theano, G., 409
Theologides, A., 281
Thiede, H. A., 165, 167
Thielgaard, A., 92
Thoma, G E., 76
Thoman, R. L , 129
Thomas, D. P., 7
Thomas, E. D., 135, 139-41
Thomas, W., 11
Thompson, F. D., 403
Thompson, H. E., 170
Thompson, J. H., 12
Thompson, R. B , 203
Thompson, W. M. Jr., 146
Thomson, J. M , 4, 11
Thomson, P. D., 401
Thor, D. E., 195
Thorburn, G. D., 373
Thrasher, K., 130, 414
Thurau, K., 368, 373
Thurlbeck, W. M., 120, 121
Thurman, G. B., 141

Thurston, J., 392
Thyrum, P. T., 400
Tibblin, G., 122, 127
Tietze, F., 309-11
Tildon, J. T., 276-78
Tillett, W. S., 142
Timothy, M. M., 83-85
Tischler, V., 316
Titus, E., 380
Tjio, J. H., 67
Tognella, S., 284
Tolis, G., 260
Tolnai, G., 202
Tomasi, T. B. Jr., 85, 188
Tomasi, V., 239, 357
Tomonoga, M., 75
Tompkins, E., 76
Tooley, W. H., 84
Topilsky, M., 198
Topper, Y. J., 237
Torelli, G., 366
Torr, J. B. D., 92
Torretti, J., 376, 379, 381
Tough, I. M., 79
Touraine, J. C., 191, 192
Tourville, D., 85
Toyoda, S., 75
Trager, W. F., 394
Travadi, V., 4
Trebilcock, H., 2
Triantaphyllopoulos, D. C., 338
Triantaphyllopoulos, E., 338
Trijbels, J. M. F., 44, 54
Trojanova, M., 278
Troll, W., 72
Trolle, D., 36
Trop, D., 117, 118
Trueheart, R. P., 145
Trygstadt, C. W., 341
Tsai, I., 389
Tsang, R. C., 319, 320, 322
Tsao, H. S., 219, 221, 223, 224
TSCHOPP, T. B., 1-18
Tschudy, D. P., 246
Tsuchiya, T., 391, 392
Tsurahara, T., 296, 297
Tsushima, T., 360
Tubiana, M., 329, 331, 348, 349
Tuck, D., 408, 409
Tuleuski, V. G., 337, 341
Tullis, J. L., 340, 341
Turck, M., 83
Turk, J. L., 179
Turkington, R. W., 237, 255, 256, 259, 260, 263, 265-67
Turnbull, A. C., 169
Turner, J. M., 115, 116, 122, 126-29
Turner, R. C., 314
Turpie, A. G. G., 10, 338
Turpin, R., 67
Tyan, M. L., 138
Tyler, F. H., 223
Tyler, J. M., 128
Tyson, J. E., 258-62

U

Uchida, I. A., 67, 68
Uhlendorf, B. W., 51
Ulfelder, H., 414
Ullman, E. F., 159
Ullrich, K. J., 374, 375
Ultmann, J. E., 325, 326, 332
Ulutin, O. N., 13
Underhill, R. A., 166, 169
Underwood, A. H., 375
Underwood, L. E., 238, 266
UNGER, R. H., 303-8; 303-6, 310, 311
Ungerstedt, U., 213
Urbach, F., 199
Urbaitis, B. K., 377, 378, 381
Uretsky, N. J., 213
Usher, R., 172
Ussing, H. H., 20
Utiger, R. D., 261, 353, 355, 356
Uylangco, C., 105

V

Vaheri, A., 5
VAITUKAITIS, J. L., 295-302; 295-99
Valdimarsson, H., 193
Vale, W., 353, 354, 357, 358
Valentine, F. T., 176
Valeriote, F. A., 332, 350
van Bekkum, D. W., 137
Vance, V. K., 368, 373-76
Vandenbroucke, J., 7
Van der Horst, R. L., 149
VanderLaan, W. P., 254, 267
Vander Stoep, L. R., 89, 90
van der Zee, S. P. M., 44, 54
van de Woestijne, K. P., 114-16, 122-24, 126
Van Gemund, J. J , 90
Van Hall, E. V., 296
van Heyningen, W. E., 19
van Hooft, J. I. M., 137
Vanikiati, B., 103
Vanky, F., 202
Van Nevel, C., 56
VanPraagh, R., 61
Van Scott, E. J., 345-48
Van Woert, M. H., 210, 211, 213
Van Wyk, J. J., 238, 266
Varga, L., 260
Vartanyan, M. E., 91, 92
Vassanelli, P., 30
Vaughan, J. H., 401
Vaughan, M., 20, 233
Veall, N., 104
Veerkamp, F. J., 296
Vekemans, M., 255, 257, 258
Velasco, J., 350
Vera, R., 325, 326
Vermylen, C., 7
Vermylen, J., 6, 7, 335
Verstraete, M., 6, 7, 335
Vertes, V., 227
Vesell, E. S., 390, 409
Vessey, M. P., 11
Vest, M. F., 35
Vidal, G., 92, 93
Videbaek, A., 77
Vieweg, W. V. R., 393
Vieyra, A., 378
Vigliani, E. C., 79
Viljanen, A., 130
Vincent, N. J., 114, 119, 125, 126
Vinciguerra, V., 331
Vinther, A., 117
Vlad, P., 61
Virchow, R., 103
Vivier, F. S., 317
Vogel, J. M., 327
Vogt, M., 212
Voisin, G. A., 138
von Bahr, C., 398
von Hayek, H., 113
Von Kaulla, E., 11
Von Kaulla, K. N., 11
von Schreeb, T., 202
Voorhees, J. J , 349
Vorne, M., 32, 33
Vosa, C. G., 68
Vosti, K. L., 83, 325
Vyas, G. N., 189

W

Wachsman, S. J., 7, 8
Waddell, W. R., 310, 313, 314
Wade, A. P., 165
Wadstrom, L. B., 202
Wagner, H., 360
Wahren, J., 369-73
Waite, B. M., 169
Waksman, B. H., 179
Walder, B. K., 79
Waldmann, T. A., 188, 191
Walker, W., 168
Wallace, C. K., 104, 105
Wallace, E. M., 79
Wallace, H. W., 242, 247
Wallach, R. G., 162
Walle, T., 402
Wallen, P., 336
Walls, W. D., 7
Walsh, P. N., 3, 5
Walters, M. N. I., 139
Waltman, R., 37
Walzak, M. P., 41, 45

Wang, B. C., 127
Wang, C., 146
Wang, C.-I., 316
Wang, H. C., 70
Wang, J. J., 332
Warburton, D., 70
Ward, D. N., 296-99
Ward, P. A., 179
Warner, A., 155, 157
Warner, G. A., 202
Warner, K., 13
Warrell, D. A., 130
Warren, S. K., 392
Washington, O., 107
Wasserman, A. J., 321
Watkins, S. P. Jr., 1
Watson, M., 390
Watson, R. A., 93
Watten, R. H., 103
Waugh, W. H., 372
Wayburne, S., 316
Ways, P., 316
Wayson, N. E., 199
Weaver, J. A., 8
Webb, J., 76
Weber, K., 338
Weber, W. W., 390
Webster, B. R., 357
Webster, J., 332
Webster, L. T., 391
Wedgwood, R. J., 192
Wegmann, T. G., 92
Wegrzynowicz, Z., 337
Weibel, E. R., 113, 119
Weidemann, M. J., 234, 369, 370, 374, 376
Weiner, I. M., 368
Weiner, J. M., 1, 2
Weiner, M. W., 376
Weingartner, H., 209, 212
Weinmann, R. L., 210
Weinschelbaum de Jairala, S., 378
Weinsier, R. L., 366
Weinstein, E., 376, 379, 381
WEINSTEIN, G. D., 345-52; 345-51
Weinstein, S. W., 368, 369, 375, 377
Weinstock, B., 249
Weintraub, M. I., 213
Weisblum, B., 68
Weiss, C. F., 35, 398
Weiss, H. J., 1, 2, 4, 5
Weissmann, G., 72
Welch, J. P., 93
Welland, F. H., 246
Wells, R., 9
Weng, J. J., 275, 276, 278
Wenger, N. K., 145, 150
Wengle, B., 32
Wenner, R., 260
Werk, E. E., 130, 414
Werk, E. E. Jr., 399
Werner, I., 353
Wertelecki, E., 141
Wesson, L. G., 366
West, J. B., 117, 121, 124
Weste, S. M., 91
Westerholm, B., 11
Weston, J. K., 35
Wetstone, H. J , 316
Wharton, R. N., 412
Whatmore, P. B., 93, 98
Wheeless, C. R., 294
Whipp, S. C , 106, 107
Whitcomb, M. E., 327
White, C. A., 168
White, J. G., 4, 5, 197
White, P., 241, 247, 248
Whitfield, C. R., 169
Whitlock, J. P. Jr., 339
Whitmore, W. F., 140
Whitsed, H. M., 12
Whittaker, J. A., 390
Whittam, R., 378, 379
Whittembury, G., 378, 379
Whyte, H. M., 316, 317
Wichmann, H. M., 34
Widdicombe, J. G., 127
Widdowson, E. M., 163
Wide, L., 296
Widen, L., 211
Wiegand, R. G., 212, 213
Wiernik, P. H., 331
Wiesner, P. J., 396
Wikler, A., 154
Wilansky, D., 252, 259, 263
WILBER, J. F., 353-64; 261, 353, 356-58, 361
Wilcox, E. B., 317
Wilcox, R. E., 114
Wilcox, W. S., 350
Wild, A. E., 168
Wiley, J. H., 94
Wilhelmsen, L., 122, 127
Wilkinson, J. F., 77
Will, J. J., 7, 8
Wille, L. E., 316
Williams, J., 95
Williams, J. D., 86
Williams, M., 198
Williams, R., 399
Williams, T. W., 179
Williams, W. J., 241, 246, 248
Williamson, D. H., 273, 275-78
Williamson, E. M., 6[illegible]
Williamson, H. E., 379, 380
Willis, J. S., 378, 379
Willoughby, J. M. T., 77
Wills, M. R., 229
Wilmore, D. W., 303, 306
Wilson, A., 167
Wilson, A. F., 123
Wilson, C. B., 149
Wilson, F. M., 149
Wilson, J. T., 31
Wilson, P. A., 13
Wilson, R., 139
Wilson, R. E., 79
Winchell, H. S., 247
Windhager, E. E., 366, 368, 379
Windmueller, H. G., 321
Winesett, P. S., 321
Winick, M., 32
Winter, J. S., 91
Winters, R. W., 368, 374, 375
Winters, W., 398
Wintrobe, M., 281-83
Wintzell, K., 327
Wise, J. B., 276, 277
Wise, J. K., 303
Witkop, C. J., 4
Wobmann, P., 8, 9
Wochner, R. D., 188, 191
Wohl, M. E., 126
Wolberg, W. H., 202
Wolf, R., 98
Wolf, A. V., 369
Wolf, K., 366, 379
Wolff, J. A., 4
Wolff, S., 8
Wolff, S. M., 197, 327
Wolfsons, S., 36
Wolstencroft, R. A., 181
Wolstenholme, G. E. W., 112, 121
Wolthmann, H. J., 233
Wong, M. Y. F. W., 167
Wood, L., 118, 119, 121
Wood, P., 61
Woodard, H. Q., 77
Woodhead, J. S., 218, 226
Woodliff, H. J., 78
Woodruff, C. W., 317
Woods, P., 317
Woodward, W. E., 105
Woolcock, A. J., 119, 123-26
Worlledge, S. M., 77
Worowski, K., 9
Wosika, P. H., 146, 148
Wotman, S., 406
Wranne, L., 244
Wray, C., 11
Wright, H. P., 13
Wright, M., 163
Wright, P. H., 309, 310, 313
Wright, S. W., 91
Wu, B. J., 106
Wybran, J., 182, 183
Wyngaarden, J. B., 48, 49, 55

Y

Yachi-Na-Songkhla, 103
YAFFE, S. J., 29-40; 30-33, 36, 37, 391, 392, 398
Yahr, M. D., 210
Yakulis, V., 201
Yalow, R. S., 217, 220, 221, 223, 224, 226-28, 234
Yamamoto, K., 89, 90
Yamamoto, T., 362

Yamatsu, K., 212
Yamazaki, H., 9, 10
Yanai, R., 265
Yankee, R. A., 197
Yao, L., 404
Yardley, J. H., 19, 26, 105
Yaryura-Tobias, J. A., 211
Yee, I. L., 316, 317
Yerushalmy, J., 172
Yeung, C. Y., 37
Ygge, H., 34
Ygge, J., 11
Yip, L. C., 48
Yokoyama, A., 4, 10
York, D., 361
Yoshida, T., 366, 373
Yoshida, Y., 89, 90
Yoshikawa, M., 222
Yoshida, T. H., 70, 72
Young, F. S., 392
Young, J. D., 194
Young, R., 218
Young, R. C., 331, 332
Young, R. R., 191
Young, W. J., 49, 50
Yu, T. F., 389
Yu, V. Y. H., 37
Yuan, L., 177
Yunis, E. J., 136, 141, 145

Z

Zacest, R., 403, 407
Zahavi, J., 10
Zapletal, A., 120, 122, 123, 126
Zaremba, J., 93
Zarembok, I., 325, 326
Zawadzki, Z. A., 77
Zeaman, D., 96
Zech, L., 67, 68, 72
Zee, P., 316
Zellweger, H., 165
Zerahn, K., 20
Zetterqvist, P., 63
Zieve, P. D., 20, 104
Zmijewski, C. M., 193
Zoschke, D. C., 140
Zublin, W., 92
ZUCKER, M. B., 1-18; 1-4, 6, 12
Zuelzer, W. W., 30
Zuirek, S. J., 168
Zuskin, E., 112, 122, 126, 127
Zweifler, A. J., 8, 13
Zweiman, B., 177, 199

# SUBJECT INDEX

A

Acenocoumarol
  drug interactions with, 394
Acetaminophen
  interactions and metabolism, 391, 392, 397, 413
Acetanilid
  neonatal metabolism of, 35
Acetoacetate
  brain utilization of, 273, 275
  renal utilization of, 370
Acetoacetyl-CoA thiolase
  on ketone utilization in brain, 376, 377
N-Acetyl-p-aminophenol sulfate conjugate
  toxicity of, 389
Actinic keratoses
  treatment of, 351
Actinomycin
  infant metabolism of, 31
Addiction
  personality aspects of, 154
Adenine phosphoribosyl transferase (APRT)
  in Lesch-Nyhan syndrome, 47, 48
Adenomas
  nonfunctional chromophobe
    prolactin-secreting, 262, 263
Adenosine diphosphate
  and platelet aggregation, 2-13
  renal, 377, 381
Adenosine monophosphate (AMP)
  renal, 377
Adenosine triphosphatase (ATPase)
  cation-stimulated
    in kidney, 378-81
  drug interactions with, 398
Adenosine triphosphate (ATP)
  in kidney, 376-78, 381
  and platelet function, 2, 4
Adenylate cyclase
  TRH activation of, 358
Adenyl cyclase
  and Clostridium perfringens infection, 107
  in intestine
    effects of cholera toxin on, 22-27, 104
Adhesiveness
  of platelets, 2
Adipose tissue
  insulin receptors in, 233-36
Adrenergic neuron
  drug interactions at, 410
Adrenocortical steroids
  drug interactions with, 395
Adriamycin
  in treatment of lymphomas, 332
Afibrinogenemia
  platelet function tests in, 5
Agammaglobulinemia
  host resistance to infection in, 293
  infantile X-linked - Bruton
    characterization of, 186-88
    treatment of, 188
  Swiss type
    treatment of, 136
Age
  and drug interactions, 390
  and airway obstruction, 122, 126, 127, 130
  gestational
    estimation of, 165-74
Agkistrodon contortrix
  venom from
    enzyme hydrolysing peptide B in, 336
Agkistrodon rhodostoma
  venom from
    as source of clotting agent, 336
Air pollution
  and airway obstruction, 127
Airway obstruction
  etiology of, 126, 127
    age, 126, 127
    environment, 127
  introduction, 111, 112

  lung function tests, 122-26
    airway resistance, 123, 124
    distribution of ventilation, 125, 126
    elastic recoil pressure, 124, 125
    forced expiratory maneuvers, 122, 123
  mechanics of air flow in, 111-34
  normal airway function, 112-19
    distribution of ventilation, 117-19
    expiration, 115-17
    extrathoracic airways, 113, 114
    inspiration, 114, 115
    intrathoracic airways, 114-19
  pathophysiology of, 119-22
    extrathoracic, 119
    intrathoracic, 119-22
  summary, 130, 131
  treatment of, 127-30
    bronchitis, 128, 129
    bronchospasm, 129, 130
    emphysema, 128
Alcohol (ethanol)
  withdrawal treatment, 157
Alcohol dehydrogenase
  in fetus, 33
Alcoholism
  chronic
    platelet function in, 7
  cirrhotic liver of
    drug interactions in, 401
Alkaline phosphatase
  in estimation of gestational age, 167
Alkali therapy
  of Lesch-Nyhan syndrome, 57
Alkylating agents
  in treatment of Hodgkin's disease, 327-31
  in treatment of lymphomas, 332
Allergy
  and airway obstruction, 127
Allopurinol
  drug interactions with, 389, 395
  in Lesch-Nyhan treatment, 56, 57
Alpha cell function, 303-8
  in diabetes mellitus, 305, 306
  normal, 303-5
  in stressful illness, 306, 307
Alprenolol
  on bronchospasm, 130
p-Aminobenzoate
  on cerebral glucose utilization, 272
p-Aminobenzoic acid
  neonatal metabolism of, 34, 35
gamma-Aminobutyric acid
  on brain glucose utilization, 278
Aminopyrine
  fetal metabolism of, 32, 33
  infant metabolism of, 27
p-Aminosalicylic acid (PAS)
  drug interactions with, 396
Amitriptyline
  drug interactions with, 409, 410, 412
Ammonia production
  renal
    and gluconeogenesis, 373
  review of, 366
Amniocentesis
  to diagnose Lesch-Nyhan syndrome, 51, 52
  risks of, 167
    ultrasound as aid in lowering risks, 169
Amniotic fluid
  prolactin levels in, 257, 258
Amniotic fluid analysis
  in estimation of gestational age, 167-69
    creatinine, 168
    cytology, 167, 168
    lecithin, 168, 169
Amobarbital
  on renal metabolism, 368
Amphetamines
  and L-dopa, 213
  drug interactions with, 409-12
Amphotericin B
  in immune deficiency treatment, 194
Ampicillin
  drug interactions of, 387
Analgesics
  interactions of, 391-93
Ancrod (Arvin)
  clotting role of, 336
Androgens
  and leukemogenesis, 78
  in treatment of Down's syndrome, 98, 99
Anemias
  aplastic
    immunologic reconstitution in, 135
    and leukemia, 78, 79
    resistance to infection in, 293
  hemolytic
    and resistance to infection, 293
    $V_{CO}$ in, 246, 247
  idiopathic aregenerative
    immunologic reconstitution in, 140
  megaloblastic
    drug interactions in, 399
Aniline
  fetal metabolism of, 33
  infant metabolism of, 31
Animals
  effects of L-dopa in, 212
Ankylosing spondylitis
  leukemogenesis by therapy in, 76
Antacids
  on chlorpromazine absorption, 408
Antiarrhythmics
  drug interactions of, 400-2, 414
Antibiotics
  in preventive management in surgery, 289-94
Anticoagulants
  fibrinogen degradation products, 338
  interactions with other drugs, 393-97, 414
    coumarin, 393-97
    heparin, 393
  in platelet function tests, 1
Anticonvulsants
  drug interactions with, 397-99, 414
Antigen-dependent migration inhibitory factor
  in immune response, 178, 179
Antigens
  table of, 180
Antihemophilic factor (Factor VIII)
  and platelet function tests, 5
Antihypertensives
  drug interactions, 407
    guanidiniums, 410, 411
  on prolactin levels, 263
Antimicrobials
  drug interactions of, 387
Antimycin
  on renal metabolism, 368
Antipyrine
  drug interactions with, 390, 409
Antiviral factors
  in immune response, 178, 179
Appendicitis
  ruptured
    hazard of infection in surgery for, 293
Arginine
  on cerebral glucose utilization, 272
Arousal
  L-dopa role in, 213, 214
Arrhythmias
  myocarditis in etiology of, 146
Arteries
  transposition of

  genetics of, 62
Ascorbic acid
  drug interactions with, 395
Aspirin
  on bleeding time, 1, 4
  drug interactions of, 388, 392, 393
Asthma
  airway obstruction in, 111, 112, 122, 124-27, 130
Ataxia telangiectasia
  site of defect in, 185, 187, 191
  therapy of, 191
Atherosclerosis
  platelet function in, 9-11
Athetoid dysarthria
  in speech of Lesch-Nyhan syndrome, 42
Atrial septal defect
  genetics of, 63
Atrioventricular valve anomalies
  genetics of, 63, 64
Atropine
  on airway obstruction, 112, 130
8-Azaguanine
  in Lesch-Nyhan syndrome, 51
Azathioprine
  drug interactions with, 389
  on flora of urethra, 85
Azide
  on renal metabolism, 368

B

Bacillus Calmette Guerin (BCG)
  in treatment of chronic myelocytic leukemia, 286
  in tumor therapy, 202-4
Barbiturates
  dependence on, 157
  drug interactions with, 395, 412
Basophilia
  in leukemia, 285
Behavior
  chromosomal aspects of, 89-102
    conclusions, 99
    methodological and conceptual issues, 95-99
    specific disorders, 89-95
  effects of L-dopa on, 209-14
Benzene
  chromosomal effects of, 79
Benzodiazepines
  drug interactions of, 397
3,4-Benzpyrine
  fetal metabolism of, 32
Bicarbonate
  effects of cholera toxin on movement of, 19, 20, 103
Bile salts
  secretion of
    drugs interfering with, 389
Biliary atresia, congenital
  hyperlipemia in, 318
Bilirubin
  and CO production, 241, 245-48
  drug interactions of, 392
Bioassays
  for prolactin, 255
Biotransformation
  and drug interaction, 288, 289
Birth
  on TRH secretion, 356
Birth defects
  congenital heart disease, 61-66
Birthweight
  and gestational age, 171
Bishydroxycoumarin
  drug interactions with, 394, 396, 409
Bladder
  infection of, 83-87
    hydrodynamics of, 85, 86
Bleomycin
  in treatment of lymphomas, 332
Blood type
  complete switching of, 136
Bone marrow
  radiation- or drug-induced failure of
    treatment of, 140
  radiotherapy-induced depression of, 327
Bone marrow aplasia
  as complication of leukemia chemotherapy, 281
  and leukemogenesis, 80
Bone marrow transplant (BMT)
  as immunologic reconstitution, 135-37, 192
Bradykinin
  contractile action of enhancer of, 339
Brain
  metabolism of ketone bodies by, 271-80
    conclusions, 278, 279
    enzymes of ketone utilization, 276-78
    ketone body utilization in vivo, 273-76
    metabolic substrates of normal adult brain, 271-73
Bronchial tree
  anatomical descriptions of, 113
Bronchitis
  airway obstruction in, 111, 112, 125, 130
    treatment, 128, 129
Bronchogenic carcinoma
  hCG in, 299
Bronchospasm
  airway obstruction in, 111, 119
    treatment of, 129, 130
Bulboventricular loop
  congenital anomalies of, 62
Burns, 3rd degree
  alpha cell function in, 306
Busulfan
  in treatment of myelocytic leukemia, 281-83, 285, 286
Butyrophenones
  on L-dopa, 212, 214
Byssinosis
  airway obstruction in, 111, 127

C

Caffeine
  to simulate Lesch-Nyhan syndrome, 54
Calcium
  disorders in metabolism of parathyroid hormone in, 217-32
  fetal-maternal interchange of, 229
Caloric restriction
  on $V_{CO}$, 246
Cancer
  hyperparathyroidism in, 226
  immunotherapy of, 202-4
  and prolactin, 265, 266
Cancer, breast
  prolactin levels in, 260, 264-66
Candida
  antigen, 180
Candidiasis, mucocutaneous
  characterization of, 187, 193
  therapy of, 193, 194
Capillary permeability
  effect of fibrinogen degradation products on, 339
Carbamazepine
  drug interactions with, 387
Carbenicillin
  drug interactions with, 387
Carbon monoxide
  biochemistry of oxidation of, 242
Carbon monoxide metabolism, endogenous, 241-50
  CO production and heme catabolism, 244-49
  CO production in human males, 243
  methods of measuring rate of CO production, 241-44
    rebreathing apparatus, 241-43

  summary, 249
Carboxyhemoglobin percent saturation (COHB)
  to study CO metabolism, 242-44, 247
Carcinogenesis
  transplacental induction of, 414
Carcinogens
  on heme catabolism, 245
Carcinoma
  basal cell
    cytokinetics of, 348-50
  prostatic
    platelet function in, 13
  squamous cell, in situ and invasive
    cytokinetics of, 348-50
Cardiac glycosides
  drug interactions with, 403-6, 414
Cardiomyopathy
  genetics of, 64
Cardiovascular agents
  drug interactions with, 399-406
    antiarrhythmics, 400-2
    cardiac glycosides, 403-6
    propranolol, 402, 403
Catecholamines
  L-dopa as precursor, 213
Catheterization
  and urinary tract infection, 86
CB-154
  on breast cancer, 266
C-banding
  in chromosome identification, 70
Cell cycle
  technic for determining duration of, 345
Cell separation
  techniques of, 137, 138
Central nervous system (CNS)
  dopaminergic tracts in
    role in behavior, 212, 213
Cerebral glucose utilization
  normal, 271
  obligatory, 272, 273
Cervix
  cytokinetics of, 348, 349
Chalone
  epidermal
    identification studies, 349
  in treatment of chronic myelocytic leukemia, 286
Chemotactic factor
  in immune response, 178, 179
Chemotherapy
  of skin hyperplasia, 350, 351
Children
  normal plasma lipids and lipoproteins in, 315-17
Chloral hydrate
  drug interactions with 388, 396
Chlorambucil
  in treatment of Hodgkin's disease, 329
Chloramphenicol
  in cholera, 105
  drug interactions with, 395
  and leukemia, 78, 79
  neonatal metabolism of, 35
Chlordiazepoxide
  in alcohol withdrawal, 157
  drug interactions with, 397
Chloride
  effect of cholera toxin on movement of, 19-22, 104
Chlormerodrin
  renal effects of, 380
p-Chloromercuribenzoate (pCMB)
  renal effects of, 380
p-Chlorophenylalanine
  on L-dopa, 212
Chlorothiazide
  drug interactions with, 397
Chlorpromazine
  drug interactions with, 407, 408, 413
  fetal metabolism of, 32
  neonatal effects of, 3[illegible]
  on prolactin levels, 259-61
Chlorpropamide
  drug interactions with, 396
Chlortetracycline
  drug interactions with, 395
Cholecystitis
  hazard of post-surgical infection in, 293
Cholera
  mechanisms and treatment, 103-5
Cholera toxin
  on fluid and electrolyte movement in small intestine, 19-28
    adenyl cyclase role, 22-27
    chloride secretion, 21, 22
    cyclic AMP, 20, 21
    mode of action of toxin, 24-27
    molecular mechanisms, 19
    reviews, 19
    short circuit, 20, 21
  purification of exotoxin, 19
Cholesterol
  primary hyperlipoproteinemia in infants, 315-22
Cholestyramine
  drug interactions with, 406
Choreoathetoid cerebral palsy
  in Lesch-Nyhan syndrome, 42
Chorionic gonadotropin, human (hCG)
  evaluation of, 295-300
    immunologic properties, 298-300
  use in estimation of gestational age, 166
Chromosome breakage
  and leukemia induction, 80
Chromosome disorders
  behavioral aspects of, 89-102
    conclusions, 99
    45,X females, 89-91
    47,XXX females, 91
    47,XXY males, 91-93
    47,XYY males, 93
    methodological and conceptional issues, 95-99
    trisomy-G, 93-95
Chromosome identification, 67-74
  discussion, 72, 73
  introduction, 67
  new techniques, 67-72
    Giemsa banding, 70-72
    other techniques, 72
    quinacrine fluorescence, 67-79
    staining of repetitive DNA, 70
Chromosomes, Philadelphia
  and chronic myelocytic leukemia, 281, 285, 286
  identification of, 68
Chromosomes, sex
  identification of, 68-70, 72
Cigarette smokers
  airway obstruction in, 111, 130
  drug interactions in, 390
Cinchophen
  drug interactions with, 396
Cirrhosis of liver
  host resistance to infection in, 293
Citrate
  in platelet function tests, 1, 5
  renal utilization of, 369
Clofibrate
  drug interactions with, 395
Clostridium perfringens
  enterotoxic diarrheal syndromes caused by, 107, 108

Clot retraction
  methodology, 1
Clotrimazole
  in candidiasis, 194
Coccidioides
  antigen, 180
Coccidioidomycosis
  immune deficiency in, 187, 197, 198, 200
Cold
  effect on TSH release, 356
  and renal metabolism, 368
Colestipol
  drug interactions with, 406
Collagen
  and platelet function, 2, 4, 5, 8, 10, 13
Concanavalin A
  insulin-like properties of, 237, 238
Conotruncal anomalies
  genetics of, 62
Contraceptives, oral
  on platelet function, 11
  on prolactin levels, 264
Corticosteroids
  on resistance to infection, 293
  in treatment of Hodgkin's disease, 327
Corticosterone
  and myocarditis, 149
Cortisol
  neonatal metabolism of, 35
Coumarin anticoagulants
  drug interactions with, 393-97
Coxsackie virus
  myocarditis from, 145, 146, 148, 149
Craniopharyngioma
  prolactin levels in, 263, 264
Creatinine
  in amniotic fluid
    in estimation of fetal age, 168
Cyanide
  renal effects of, 368, 377
Cyclazocine
  in treatment of heroin addiction, 160
Cyclic 3', 5'-adenosine monophosphate (cyclic AMP)
  insulin effects on, 239
  in Lesch-Nyhan syndrome, 54
  role in cholera toxin electrolyte movement, 19, 20, 22, 104
  TRH activation of, 358, 362
Cyclohexamide
  in cholera, 105
Cyclophosphamide
  bone marrow repair after treatment with, 140
  leukemogenesis by, 78
  in treatment of Hodgkin's disease, 328, 329
  in treatment of lymphomas, 332
  in treatment of skin hyperplasia, 350
Cystic fibrosis
  airway obstruction in, 111, 120, 130
Cystine amino peptidase
  in estimation of gentational age, 167
Cystitis
  causes of, 83-87
Cytochrome P-450
  in fetal and neonatal drug metabolism, 33
Cytogenetics
  chromosome identification, 67-74
Cytokinetics
  of epidermal hyperplasia, 345-52
Cytosine arabinoside
  in chronic myelocytic leukemia, 285
  in treatment of lymphomas, 332
Cytotoxic drugs
  immunodeficiency with, 135, 140
  leukemogenesis by, 77-79

D

Dapsone
  drug interactions with, 390
Death, sudden
  myocarditis as cause of, 146, 147
Decarboxylase inhibitors
  and L-dopa, 212
Deoxyribonucleic acid (DNA)
  staining of repetitive (C-banding), 70, 71
Depression
  behavioral effects of L-dopa in, 212
  and heart disease
    treatment of, 411, 412
Dermatoses, benign
  cytokinetics of, 347-49
Desalanine insulin
  binding of, 234, 235
Desasparagine insulin
  binding of, 234, 235
Desipramine
  drug interactions with, 410
Desmethylimipramine
  drug interactions with, 408, 412
Desoctapeptide insulins
  binding of, 234
Desoxycorticosterone acetate (DOCA)
  and renal substrate utilization, 372
Dexamethasone
  drug interactions with, 399
Dextroamphetamine
  drug interactions with, 412
Dextrocardia
  genetics of, 62
Dextrothyroxine
  drug interactions with, 395
Diabetes mellitus
  acidosis of
    host-susceptibility to infection, 293
  alpha cell function in, 305, 306
  cerebral ketone utilization in, 272-76
  maternal
    estimation of fetal age, 165, 168
  platelet function in, 8
Diarrhea
  enterotoxic, 103-10
    cholera, 103-5
    Clostridium perfringens, 107, 108
    Escherichia coli, 105-7
$^{125}$I-Diatrizoate (Hypaque)
  drug interactions with, 393
Diazepam
  drug interactions with, 397
Diazepoxide
  in alcohol withdrawal, 157
Diazoxide
  drug interactions with, 388, 396
Dibromodulcitol
  in chronic myelocytic leukemia, 284
Dibromomannitol
  in chronic myelocytic leukemia, 283, 284
Dicumarol
  drug interactions with, 390
Diethylnicotineamide
  neonatal metabolism of, 37
Diethylstilbestrol
  as cause of adenocarcinoma of vagina, 414
Digitalis glycosides
  radioimmunoassay for, 405
  toxicity, 405, 406
Digitoxin
  drug interactions with, 403-6
Digoxin
  drug interactions with, 403-6
  renal effects of, 379
L-3, 4-Dihydroxyphenylalanine

  see L-Dopa
2, 3-Dimercaptopropanol
  on TRH, 355
Dimethylbenzanthracene (DMBA)
  on induction of mammary tumors, 265
Dinitrochlorobenzene (DNCB)
  transfer of sensitivity to, 176
2, 4-Dinitrophenol (DNP)
  renal effects of, 376-78
d, l-4, 4-Diphenyl-6-dimethylamino-3-heptanone
  see Methadone
Diphenylhydantoin (DPH)
  drug interactions with, 387, 397-99, 404
  effects on neonate, 34
  and leukemia, 79
Diseases
  Bright's
    as TSH deficiency state, 361
  Burkitt's lymphoma
    chromosome identification in, 68
  chronic granulomatous
    site of defect, 185
    transfer factor in, 196
  glycogen storage
    hyperlipidemia in, 318
    platelet function in, 7
  Graves'
    TRH in, 361, 362
  heart
    see Heart disease
  Hodgkin's
    chemotherapy of, 281
    current concepts in treatment of, 325-31
    leukemia rate in, 76
    survival, 285
  Parkinson's
    behavioral changes after L-dopa treatment, 209-11
  Von Willebrand's
    platelet function tests in, 5
Disodium chromoglycate
  in asthma, 130
Disseminated intravesicular coagulation (DIC)
  platelet function in, 6, 7
Disulfiram
  drug interactions with, 395
Diurnal variation
  and platelet function, 304
Diverticulitis
  perforated
    hazards of post-surgical infection in, 293
L-Dopa
  mental effects of, 209-16
    animal behavior, 212
    behavioral activation, 213, 214
    biochemical basis for, 212, 213
    IQ, learning, and memory changes, 212
    in non-Parkinsonian subjects, 211, 212
    in Parkinson's disease, 209-11
    on prolactin levels, 250, 261, 263, 266
    on TSH release, 357, 362
Dopamine
  on TRH, 355
Drug addiction
  methadone treatment of heroin addiction, 153-63
Drug combinations
  in treatment of lymphomas, 328-32
Drug interactions, 385-418
  analgesics-salicylates, 391-93
  anticoagulants, 393-97
  anticonvulsives, 397-99
  antihypertensives, 407
  cardiovascular drugs, 399-406
  concluding remarks, 414
  drug levels, 390, 391
  introduction, 385
  mechanisms of drug interactions, 385-90
    action, 389
    biotransformation, 388, 389
    elimination, 389
    in vitro, 386, 387
    pharmacogenetics, 389, 390
    receptors, 388
    route, 387
    serum protein binding, 387, 388
    table of, 385
  in pediatrics, 413, 414
  psychotropics, 407-13
  what are drug interactions? 385
Dyskinesis
  and L-dopa, 211

E

ECHO virus
  myocarditis from, 145, 146, 148
Electroencephalogram (EEG)
  effects of L-dopa on, 212
Electrolytes
  cholera toxin effects on, 19-27
Electron transport system (ETS)
  renal
    natriuresis, 368
Embolism, pulmonary
  plasma fibrinogen-fibrin degradation products in, 341
Emphysema
  airway obstruction in, 111, 112, 120, 121, 130
    etiology, 126
    pathophysiology, 120, 121
    treatment, 128
Empyema
  hazard of post-surgical infection in, 293
Endocardial cushion defects
  genetics of, 63
Energy metabolism
  renal
    and sodium reabsorption, 365-84
Enterotoxic diarrheal syndromes, 103-10
  cholera, 103-5
  Clostridium perfringens, 107, 108
  Escherichia coli, 105-7
Environments
  methods of conceptualizing and assessing, 98
Enzymes
  of ketone utilization in brain, 276-78
Enzyme systems
  comparison of fetal, infant, and adult, 31, 36
Ephedrine
  drug interactions with, 410
Epidermal hyperplasia
  cytokinetics of, 345-52
    and chemotherapy, 350, 351
    comparative in vivo data - table of, 348
    in epithelial malignancies, 349, 350
    in normal skin and benign dermatoses, 347-49
    principles and methods of studying, 345, 346
Epididymal fat pads
  insulin receptor in, 233
Epinephrine
  on cerebral glucose utilization, 272
  on host resistance to bacterial infection, 291
  on insulin activity, 239
  on microsomal heme oxygenase activity, 245
  and platelet aggregation, 2-4, 7, 8, 10, 13
  in treatment of bronchospasm, 129
Ergocryptine
  on prolactin secretion, 260,

261, 263, 266

Erythroblastosis fatalis
- fetal age estimation in, 165

Erythropoiesis, ineffective
- and $V_{CO}$, 247

Escherichia coli
- enterotoxic diarrheal syndromes caused by, 105-7
- and urinary infection, 83-85

Estrogens
- drug interactions with, 395
- on platelet function, 11
- and prolactin secretion, 257, 263-66
- and TSH release, 362
- and urinary infection, 84

Ethacrynic acid
- in cholera, 104
- drug interactions with, 396

Ethanol
- drug interactions with, 390
- neonatal metabolism of, 37
- see also Alcoholism

Ethchlorvynol
- drug interactions with, 395

Ethionine
- neonatal metabolism of, 36

Ethylbiscoumacetate
- drug interactions with, 394

Ethylene diamine tetra acetic acid (EDTA)
- in platelet function tests, 1

Ethylmorphine
- fetal metabolism of, 33

Exercise
- and insulin:glucagon ratio, 304
- and myocarditis, 149
- and platelet function, 3, 4
- on prolactin levels, 259, 264

Expiration
- air flow mechanics in, 115-17

F

Familial diseases
- heart disease, 61-66

Fasting
- and mineral metabolism, 366

Fatty acids, free (FFA)
- renal utilization of, 369-71

Fetus
- drug metabolism in, 29-33
- enzyme systems in, 31, 32
- estimation of gestational age of, 165-74
  - amniotic fluid analysis, 167-69
  - assessment of specific fetal systems, 167-71
  - evaluation of duration of pregnancy, 165-67
  - introduction, 165
  - mean biparietal diameters for each week of pregnancy, 171
  - postnatal estimation of gestational age, 171, 172
  - summary, 172
  - ultrasound, 169-71
- normal plasma lipids and lipoproteins in, 315, 316
- prolactin levels in, 258
- transplantation of tissues of
  - in immunologic reconstitution, 138, 141

Fibrinogen
- concept of molecule, 335
- physical characteristics of, 337

Fibrinogen-fibrin degradation products, 335-44
- biological properties, 338, 339
- characterization and purification, 337, 338
- clinical significance, 340-42
- conclusion, 342
- methods of detection, 339, 340
- physical characteristics of, 337
- structural considerations, 335-37

Ficin
- clotting action of, 336

Flufenamic acid
- drug interactions of, 388

Fluoride
- cholera toxin effects on, 23, 24
- on insulin activity, 239

5-Fluorouracil
- in skin hyperplasia, 350, 351

Folic acid
- drug interactions with, 398

Follicle stimulating hormone, human (hFSH)
- evaluation of, 295, 296, 298
  - immunologic properties, 298, 299

Free radical assay technique (FRAT)
- in treatment of heroin addiction, 159

Furalazine
- in cholera, 105

Furazolidone
- and cholera, 105
- drug interactions with, 411

Furosemide
- on prolactin levels, 259

Fusidic acid
- drug interactions of, 387

G

Gamma-ray
- leukemogenesis by, 75

Gastrointestinal tract
- cancer of
  - hCG in, 299

G-banding
- in chromosome identification, 70-72

Genetics
- behavioral aspects of chromosomal disorders, 89-102
- chromosome identification, 67-74
- of congenital heart disease, 61-66
  - introduction, 61
  - summary, 64, 65
  - types, 61-64
- of Lesch-Nyhan syndrome, 49-53

Gentamicin
- interactions with drugs, 387

Gestational age
- estimation in fetus, 165-74
- estimation postnatally, 171, 172

Gestational choriocarcinoma
- hCG in, 299

Giardia lamblia
- in agammaglobulinemia, 186

Giemsa banding (G-banding)
- in chromosome identification, 70-72

Glomerulonephritis
- fibrinogen-fibrin degradation products in, 341
- on platelet function, 12

Glottis
- airways of, 113, 114

Glucagon
- drug interactions with, 395
- hormone role of, 303-7
- on insulin activity, 239
- on microsomal heme oxygenase activity, 245

Glucocorticoids
- drug interactions of, 387
- oral
  - on TSH secretion, 356, 357

Glucose
- cerebral utilization of
  - normal, 271, 278
  - obligatory, 272, 273
- hepatic extraction of insulin after, 311-13

  renal utilization of, 372-76, 378
Glucuronidation
  in fetus and newborn, 29, 30, 36, 37
Glutamate
  on cerebral glucose utilization, 272, 278
Glutethimide
  drug interaction with, 395
Glycine
  on cerebral glucose utilization, 272
Gonadal dysgenesis, pure
  behavioral aspects of, 89
Gonadotropic hormones
  advances in evaluation of, 295-302
    chemical properties, 295-300
    general, 295
    immunological properties, 298-300
    introduction, 295
    sialic acid, 296
    subunit structure, 296, 297
Gout
  and Lesch-Nyhan syndrome, 44, 55
Graft-versus-host reaction
  techniques of immunologic reconstitution in, 136-43
    cell separation, 137, 138
    clonal destruction, 140, 141
    immunologic enhancement, 138, 139
    leukocyte transfusions, 142
    suppression of bacterial flora, 139, 140
    thymus transplantation, 141, 142
    transfer factor, 142
Granulocytes
  chemotaxis of
    induction of, 339
  immune defects in, 185, 196, 197
Griseofulvin
  drug interactions, 387
Growth hormone, human (hGH)
  amino acid sequence of, 254
  on drug metabolism, 31
  separation from prolactin, 251-53
Guanethidine
  drug interactions with, 410, 411
Guanidinosuccinic acid (GSA)
  on platelet function, 7, 8

H

Hageman factor (Factor XII)
  deficiency of
    platelet function tests 5
Haloperidol
  drug interactions with, 413
Hamycin
  drug interactions of, 387
Heart
  defects of septation
    genetics of, 63
  viral myocarditis and its sequelae, 145-52
    arrhythmias and conduction disturbances, 146
Heart disease
  effect on resistance to infection, 293
Heart disease, congenital
  genetics of, 61-66
    anomalies of visceroatrial situs and bulboventricular loop, 62
    atrioventricular valve and semilunar valve anomalies, 63, 64
    cardiomyopathy and conduction defects, 64
    conotruncal anomalies, 62
    defects of cardiac septation, 63
    summary, 64, 65
    supravalvar anomalies, 64
    types, 61-64
Heart disease, cyanotic
  platelet function in, 7
Heart disease, ischemic
  hyperlipoproteinemia in, 318, 322
Hemangioma, giant
  platelet function in, 6, 7
Heme catabolism
  and CO production, 244-49
Hemeprotein P-450
  and drug interactions, 388, 389
Hemolysis
  CO metabolism in, 244, 246-48
Heparin
  drug interactions with, 385, 393
  on platelet function tests, 1, 5
Hepatitis
  and myocarditis, 146
Hereditary hemorrhagic telangiectasia
  platelet function in, 13
Heroin
  British system of heroin maintenance, 161, 162
  methodone treatment of addiction, 153-63
    conclusion, 163
    description of, 157-63
    introduction, 153-56
    pharmacological and medical aspects of methodone, 156, 157
Hexachlorophene
  infant toxicity of, 413, 414
Hexobarbital
  on neonate, 31, 32
Histamine
  role in airway obstruction, 126, 127
Homocystinuria
  platelet function in, 13
Hormones
  changes in at birth and weaning, 30
Hydralazine
  drug interactions with, 390, 407
β-Hydroxybutyrate
  renal utilization of, 370
D(-)-β-Hydroxybutyrate
  brain utilization of, 273, 275, 276
3-Hydroxybutyrate dehydrogenase
  on brain ketone utilization, 276, 277
5-(p-Hydroxyphenyl)-5-phenylhydantoin (HPPH)
  drug interactions with, 398
Hydroxyurea
  in chronic myelocytic leukemia, 283, 284
  in skin hyperplasia, 350
Hyperbilirubinemia
  drug interactions in, 387
  enzyme induction in, 36, 37
Hypercalcuria, idiopathic
  circulating parathyroid hormone in, 229
Hypercholesterolemia, familial
  description of, 318-20
Hyperchylomicronemia
  familial lipoprotein lipase deficient
  characterization of, 321, 322
Hyperglucagonemia
  function of, 304, 306
Hyperglycemia
  alpha cell function in, 304-7
  on hepatic insulin extraction, 313
Hyperkeratosis, epidermolytic
  cytokinetics of, 348
  treatment of, 250
Hyperlipemia
  on platelet function, 9
Hyperlipoproteinemia, primary infantile, 318-22

  Type I, 321, 322
  Type II, 318-21
    description of, 318-20
    detection of, 320
    treatment of, 320, 321
  Types III, IV, and V, 322
Hyperparathyroidism, ectopic
  circulating parathyroid hormone in, 226, 227
Hyperparathyroidism, primary
  circulating PTH in, 224-26
    adenoma vs hyperplasia, 225
    effect of parathyroidectomy, 226
    preoperative localization, 225, 226
    steady-state concentrations, 224, 225
Hyperparathyroidism, renal
  circulating PTH in, 227, 228
Hypersplenism
  and resistance to infection, 293
Hypertension
  maternal
    estimation of fetal age, 165
  platelet function in, 13
Hyperthyroidism
  and leukemogenesis, 76
  TRH in, 361, 362
Hypertrophic subaortic stenosis
  genetics of, 64, 65
Hyperuricemia
  in Lesch-Nyhan syndrome, 43, 56
Hypoalbuminemia
  drug interactions in, 387
Hypogammaglobulinemia
  non-sex-linked adult, 189, 190
  transient, infant, 188, 189
  X-linked infantile
    site of defect in, 185, 187
Hypoglycemia
  cerebral glucose utilization in, 272
Hypomania
  from L-dopa, 212
Hypoparathyroidism
  circulating parathyroid hormone in, 223
Hypoprothrombinemia
  drug-induced, 395, 396
Hypothalamic disorders
  prolactin levels in, 264
Hypothalamus
  and thyrotropin-releasing hormone, 355
Hypothyroidism
  following radiotherapy, 327
  prolactin levels in, 264
  TRH in, 360, 361
Hypoxanthine guanine phosphoribosyl transferase (HGPRT)
  in Lesch-Nyhan syndrome, 41, 47-58
  molecular heterogeneity of, 54-56
Hypoxia
  and myocarditis, 149

I

Ichthyosis, lamellar
  cytokinetics of, 348
  treatment of, 350
Ichthyosis, sex-linked
  cytokinetics of, 348
Ichthyosis vulgaris
  cytokinetics of, 348
Imipramine
  drug interactions with, 410, 412
Immune deficiency states
  transfer factor therapy in, 184-204
    acquired, 197-204
    congenital or hereditary disorders, 186-97
    development of immune response, 184-86
Immune response
  development of, 184-86
    schematic of, 185
    site of, 185
  factors secreted by activated lymphocytes in, 178
Immunity
  assessment of, 179-84
    antigens, table of, 180
    methods, 180
    schematic of assay for lymphocyte transformation, 181
    schematic of assay for MIF, 182
    typical MIF patterns, 183
  in vitro transfer of, 177
Immunization
  in treatment of chronic myelocytic leukemia, 286
Immunoassay
  of fibrinogen-degradation products, 339, 340
  for insulin, 310, 311
Immunoglobulin A (IgA)
  in urinary tract, 85
Immunoglobulin G (IgG)
  in bladder, 85
Immunoglobulins
  in immune response, 178, 179
  selective disorders of, 185, 188, 189
Immunologic disorders
  platelet function tests in, 5, 6
Immunologic reconstitution, 135-44
  immunodeviation and modulation of graft-vs-host disease, 137-43
    cell separation, 137, 138
    clonal destruction, 140, 141
    immunologic enhancment, 138, 139
    leukocyte transfusions, 142
    suppression of bacterial flora, 139, 140
    thymus transplantation, 141, 142
    transfer factor, 142
  introduction, 135-37
  summary, 142, 143
Immunology
  properties of gonadotropins, 298-300
  transfer factor therapy in immune deficiency states, 175-204
Immunosuppression
  and leukemogenesis, 80
Immunosuppressive therapy
  immune deficiency state from, 187, 204
Impotency
  behavioral effects of L-dopa in, 211
Incontinent nostalgia
  from L-dopa, 212
Indomethacin
  drug interactions with, 392, 396
Infant
  cerebral ketone metabolism in, 275
  drug metabolism in, 33-37
  premature
    prolactin levels of, 257
  primary hyperlipoproteinemia in, 315-24
    familial type I, 321, 322
    familial type II, 318-21
    normal plasma lipid and lipoproteins, 315-17
    voids in current knowledge, 322
Infection
  alpha cell function in, 306
Influenza virus
  myocarditis as sequel of, 146
Insecticide
  drug interactions with, 390
Inspiration
  air flow mechanism in, 114, 115
Insulin

  binding of, 233
  hepatic extraction of, 309-14
    factors regulating, 313, 314
    general comments, 314
    after glucose, 311-13
    measurement of, 309-11
  in normal alpha cell, 303-5
  receptors, 233-40
    effects of enzyme digestions of cell surface, 236, 237
    effects of insulin-agarose derivatives, 237
    insulin, mitogens, and cell growth, 238
    isolation and purification of receptor, 238, 239
    measurement of receptor interactions, 233-35
    mechanism of insulin action, 239
    and plant lectins, 237, 238
    receptors in insulin resistant states, 237
    subcellular localization of, 235, 236
    summary, 239
$^{131}$I-Insulin
  to measure basal hepatic extraction of insulin, 309, 310
Insulin:glucagon ratio (I/G)
  and alpha cell function, 304-7
Intelligence quotient (IQ)
  chromosomal disorders influencing, 89-99
  effects of L-dopa on, 212, 213
Interferon
  in immune response, 178, 179, 198
Intestinal obstruction
  host resistance to infection in, 293
Intestine, small
  action of cholera toxin on fluid and electrolyte movement in, 19-28
Intrauterine diagnosis
  of Lesch-Nyhan syndrome, 51, 52
Iodoacetamide
  diuretic action of, 275
Iodoinsulin
  binding of, 234, 235
Isoniazid
  drug interactions with, 390, 395
Isoproterenol
  in treatment of cystic fibrosis, 120

J

Jaundice
  neonatal
    drug metabolism role in, 34
    treatment of, 36, 37
Jejunum
  cholera toxin effects on, 24

K

K-88 antigen
  and pathogenicity, 107
3-Ketoacid-CoA transferase
  on ketone utilization in brain, 276, 277
Ketoacidosis
  insulin therapy of, 306
Ketone bodies
  brain metabolism of, 271-80
    conclusions, 278, 279
    enzymes of ketone utilization, 276-78
    ketone body utilization in vivo, 273-76
    metabolic substrates of normal adult brain, 271-73
  renal utilization of, 37[illegible]
Kidney
  advanced renal failure
    prolactin levels in, 263, 264
  energy metabolism and sodium reabsorption in, 365-84
    ATP in kidney, 376-78
    cation-stimulated ATPase, 378-81
    introduction, 365, 366
    metabolism and function of renal medulla, 373-76
    renal sodium reabsorption and oxygen consumption, 368, 369
    and substrate utilization, 369-73
  fibrin deposition in, 34[illegible]
  renal insufficiency
    parathyroid hormone in, 227
Kveim antigen
  in sarcoidosis, 199, 200

L

Lactate
  on cerebral glucose utilization, 273
  renal utilization of, 371, 372, 375
Lactation
  hormones required for, 266, 267
Learning
  effects of L-dopa on, 212, 213
Lecithin
  in amniotic fluid
    in estimation of gestational age, 168, 169
Lectins
  of plants
    and insulin receptors, 237, 238
Leprosy
  immune deficiency in, 199
  transfer factor therapy of, 199, 200
Leukemia
  platelet function in, 12
  radiation and drug therapy as cause of, 75-82
    Atomic Bomb Casualty Commission studies, 75, 76
    cytotoxic drugs, 76-78
    discussion, 79, 80
    mechanisms of therapy-related induction of, 80
    other therapeutic drugs, 78, 79
    summary, 81
    X-ray, 76, 77
  resistance to infection in, 293
  transfer factor therapy of, 203
Leukemia, chronic myelocytic
  current concepts in treatment of, 281-88
    alternative antileukemic drugs, 284
    common complications, 284, 285
    conclusions, 287
    conventional therapy and course, 281-84
    new approaches to therapy, 285, 286
Leukemia, chronic myeloid
  chromosome identification in, 68
Leukemia, lymphocytic
  insulin receptors in, 238
Leukocytes
  transfusions of, 142
Lidocaine
  drug interactions with, 401, 402
Lipids, plasma
  evolution of, 317
Lipoproteins
  high density (HDL), 315-17, 319
  low density (LDL), 315-21

  very low density (VLDL), 315-19
Liver
  insulin extraction by, 309-14
    factors regulating, 313, 314
    general comments, 314
    after glucose, 311-13
    measurement of, 309-11
  insulin receptor in, 233-35
Liver disease
  platelet function in, 7
Lung
  estimation of fetal maturity of, 169
  function tests of, 122-26
    airway resistance, 123, 124
    distribution of ventilation, 125, 126
    elastic recoil pressure, 124, 125
    forced expiratory maneuvers, 122, 123
  mechanics of airway obstruction, 111-34
Luteinizing hormone (hLH)
  evaluation of, 295-99
    immunologic properties, 298-300
Lymph nodes
  excision of
    post-surgical infection hazard, 293
Lymphocytes
  assay for transformation of, 181
  factors secreted by in immune response, 178
  insulin binding by, 238
Lymphoma
  current concepts in treatment of, 325-34
    Hodgkin's disease, 325-31
    other, 331-33
  following anticonvulsant therapy, 399
  hCG in, 299
Lymphoreticular malignancy
  causes of, 79
  immune deficiency in, 187
Lymphosarcoma
  and leukemia, 77
  treatment of, 331
Lymphotoxin
  in immune response, 178, 179

M

Macroglobulinemia
  on platelet function, 12
Macrophage activation factor
  in immune response, 178, 179
Macrophage aggregation factor (MAF)
  in immune response, 178, 179
Magnesium
  on parathyroid hormone secretion, 220
Malignancy
  cellular immune defects in, 135, 185, 187
    transfer factor therapy of, 201-4
Malonate
  on renal metabolism, 368
Maltose
  cerebral utilization of, 272
Mammary gland
  insulin receptor in, 233
  prolactin effects on, 266, 267
Manic-depressive state
  L-dopa role in, 214
Mannose
  cerebral utilization of, 272, 273
Medulla, renal
  metabolism and function of, 373-76
Mefenamic acid
  drug interactions with, 396
Melanoma, malignant
  cytokinetics of, 348
Melphalan
  leukemogenesis by, 77, 78
Memory
  effects of L-dopa on, 212, 213
Menstrual cycle
  prolactin levels in, 258
Mental retardation
  chromosomal disorders, 89-99
  in Lesch-Nyhan syndrome, 41, 55
Meprobamate
  drug interactions of, 396, 397
Meralluride
  renal effects of, 380
6-Mercaptopurine (6-MP)
  in chronic myelocytic leukemia, 283-85
  drug interactions with, 395
  in Lesch-Nyhan syndrome, 51
Metaproterenol
  in bronchospasm, 129
Metaraminol
  drug interactions with, 410-12
Methadone
  pharmacological and medical aspects of, 156, 157
  in treatment of heroin addiction, 153-64
    conclusions, 163
    description of, 157-63
    evaluation of, 158, 159
    introduction, 153-56
    treatment goals, 162, 163
    urine monitoring, 159
Methadyl acetate
  in treatment of heroin addiction, 160
Methotrexate
  drug interactions of, 392
  in graft-versus host reaction, 140
  in skin hyperplasia, 350
  in treatment of Hodgkin's disease, 329
  in treatment of lymphomas, 332
4-Methylcoumarin hydroxylase
  in newborn and infant, 30
Methyldopa
  on prolactin levels, 263
α-Methylparatyrosine
  and L-dopa, 212
Methylphenidate
  drug interactions of, 412
Methyl polysiloxane
  drug interactions with, 395
Methylpurines
  and Lesch-Nyhan syndrome, 53
Metropathia hemorrhagica
  leukemia rate in, 76
Micropuncture
  to study renal sodium reabsorption and energy metabolism, 366
Microsomal heme oxygenase (MHO)
  characterization of, 244, 245, 248, 249
Micturition
  anti-infective role of, 85, 86
Migraine
  behavioral effects of L-dopa in, 211
Migration inhibitory factor (MIF)
  in immune response, 178-84, 194
Mitogenic factor
  in immune response, 178
Mitogens
  and cell growth, 238
Monoamine oxidase
  and platelet function, 13
Monoamine oxidase inhibitors (MAOI)
  and L-dopa, 212
  drug interactions with, 396,

409, 412
Mononucleosis
  cardiac sequelae of, 146, 148
Monosodium glutamate
  in treatment of Lesch-Nyhan syndrome, 58
MOPP program
  in treatment of Hodgkin's disease, 328, 329, 331
Multiple sclerosis
  platelet function in, 11, 12
Mumps
  antigen, 180
  heart disturbances following, 146, 149
Myeloma, multiple
  leukemogenesis by therapy of, 77, 78
  platelet function in, 12
Myeloma, plasma cell
  leukemia association with, 77, 78
Myelomatosis
  leukemogenesis in, 77
Myeloproliferative disorders
  platelet function tests in, 12
Myocardial infarction, acute
  platelet function tests in, 5, 9, 10
Myocarditis, viral
  see Viral myocarditis
Myxedema
  TRH in, 360

N

NADPH cytochrome c reductase
  in neonate and infant, 33
Nalidixic acid
  drug interactions with, 396
  neonatal metabolism of, 35
Nalorphine
  in heroin overdose treatment, 157
Narcolepsy
  L-dopa in treatment of behavioral effects of, 211
Narcotics
  rehabilitation of addicts
    methadone treatment of heroin addiction, 153-63
Necrotizing factor
  in immune response, 178, 179
Neomycin
  drug interactions with, 392, 395, 396
Neonate
  detection of hyperlipoproteinemia in, 320
Neoplasia
  incidence of hCG in, 299, 300
Neuraminidase
  on insulin receptor, 233, 238
Neutrons
  leukemogenesis by, 75
Newborn
  drug metabolism in, 28-40
    animal studies, 29-32
    conclusion, 37, 38
    human fetus, 32, 33
    human neonate and infant, 33-37
    induction of drug metabolizing activity, 35-37
  estimation of gestational age of, 171, 172
  prolactin levels of, 257, 264
  respiratory distress in
    prevention of, 168, 169
  treatment of drug addiction in, 157
Nitrazepam
  drug interactions with, 397
p-Nitroaniline
  fetal metabolism of, 32
p-Nitroanisol
  fetal metabolism of, 32
p-Nitrobenzoic acid
  fetal metabolism of, 32
Nitrogen
  to study distribution of ventilation, 125
Nitrogen mustard
  in treatment of Hodgkin s disease, 328, 330
  in treatment of skin hyperplasia, 350
Nitroglycerine
  importance of glass container for, 387
Nitrous oxide
  drug interactions with, 390
Norepinephrine
  drug interactions with, 410, 411
  and platelet function testing, 5, 10, 11
  on thyrotropin-releasing hormone, 355
Nortriptyline
  drug interactions with, 390, 395, 409, 413
Nose
  airways of, 113, 114
Nursing mothers
  prolactin levels of, 257 264

O

Oleate
  renal utilization of, 369
Oligomycin
  renal effects of, 378
Oral contraceptives
  see Contraceptives, oral
Osteitis fibrosa
  hypercalcemia of, 228
Osteogenesis imperfecta
  platelet function in, 13
Ouabain
  on renal Na-K-ATPase, 378, 379, 381
Ovarian dysgenesis
  behavioral aspects of, 89-91
7-Oxa-13 prostynoic acid
  on TRH-mediated TSH release, 358
Oxygen
  to treat airway obstruction, 128
Oxygen consumption
  and renal sodium reabsorption, 366-69, 374
    effect of metabolic inhibitors, 368, 369
    effect of physiologic maneuvers, 367, 368
Oxyphenbutazone
  drug interactions with, 396
Ozone
  on lung function, 127

P

Palmitate
  renal utilization of, 369, 374, 375
Pancreas
  alpha cell function, 303-7
Pancytopenias
  immunologic reconstitution in, 140
Papain
  clotting action of, 336
Parathyroid aplasia
  treatment and characterization of, 190
Parathyroid hormone (PTH)
    in calcium disorders, 217-32
  basic considerations, 218-20
    biosynthesis, 219
    chemistry, 218-29
    secretion, 219, 220
  circulating PTH, 223-29
    ectopic hyperparathyroidism, 226, 227
    hypercalcuria, 229
    hypoparathyroidism, 223
    normal, 223
    pregnancy, 228, 229
    primary hyperparathyroidism, 224-26
    pseudohypoparathyroidism, 224

    renal hyperparathyroidism, 227, 228
    rickets, 228
    sarcoidosis, 229
  radioimmunoassay, 220-23
    immunochemical heterogeneity of circulating PTH, 221, 222
    problems in interpretation, 222, 223
    technical aspects, 220, 221
Paregoric
  in treatment of neonatal withdrawal syndrome, 157
Pediatrics
  drug interactions in, 413, 414
Penicillin
  drug interactions of, 392
  preventive use in surgery, 289
Pentazocine
  drug interactions with, 390
Perphenazine
  drug interactions with, 413
Pethidine
  neonatal effects of, 34
Phalloidin
  newborn tolerance of, 32
Pharmacogenetics
  of drug interactions, 389, 390
Pharmacology
  drug interactions, 385-418
Phenacetin
  drug interactions with, 390
Phenelzine
  drug interactions with, 390
Phenformin
  drug interactions with, 392
Phenobarbital
  and bronchospasm, 130
  drug interactions with, 387, 392, 397, 398, 402, 404, 413, 414
  in heme metabolism, 245, 246
  neonatal metabolism of, 36, 37
Phenobarbitone
  in treatment of infant jaundice, 37
Phenocoumarin
  drug interactions with, 394
Phenothiazines
  and L-dopa, 212, 214
  drug interactions with, 407, 408, 411, 412, 414
  on prolactin levels, 259-61, 264
Phenylbutazone
  and leukemogenesis, 78, 79
  neonatal metabolism of, 37
Phenylketonuria
  offspring of phenylketonuric mothers
    heart defects in, 62
Phenyramidol
  drug interactions with, 395
Phosphodiesterase
  on intestinal cyclic AMP, 22
Phosphoglycerate kinase (PGK)
  renal catalytic role of, 381
Phospholipases C or A
  on insulin binding, 236, 238
5-Phosphoribose-1-pyrophosphate (PRPP)
  in Lesch-Nyhan syndrome, 48, 49
$^{32}$Phosphorus
  and leukemogenesis, 77
Phylloquinone oxide
  drug interactions with, 394
Pituitary tumors
  prolactin-secreting, 259, 262-64
  TRH effects on, 361, 362
    in euthyroids, 362
Pityriasis rubra pilaris
  therapy of, 350
Placenta
  method of locating, 169
Placental lactogen, human
  amino acid sequence of, 254
Plasma protein
  congenital deficiencies in
    platelet function tests, 5
Platelet factor 3 (PF-3)
  in platelet function tests, 3, 6-9, 12, 13
Platelet function
  effect of fibrinogen degradation products on, 338
Platelet function tests, 1-18
  acquired disorders of function, 5-13
  congenital disorders due to abnormal platelets, 4
  congenital disorders due to plasma protein deficiencies, 5
  methodology, 1-4
    ADP and aggregation, 2
    bleeding time, 1
    clot retraction, 1
    diurnal variation, epinephrine administration and exercise, 3, 4
    electrophoretic mobility, 3
    platelet age and function, 3
    platelet factor, 3, 8
    platelet release reactions, 2
    technical difficulties, 1
    tests of adhesiveness and retention, 2, 3
  summary, 13
Pneumonia
  platelet function tests in, 5
Poliomyelitis virus
  myocarditis from, 145, 150
Polycythemias
  leukemogenesis by radiotherapy in, 76, 77
  platelet function in, 7
Postpartum women
  prolactin levels in, 257, 264
Potassium
  and cholera, 103
  chronic deficiency of
    on renal oxygen consumption, 376
PPD antigen
  in assessment of immunity, 180, 202
Prednisolone
  on kidney, 380
Prednisone
  drug interactions of, 388
  on insulin receptor, 237
  leukemogenesis studies, 78
  to treat airway obstruction, 128, 130
  in treatment of Hodgkin's disease, 328, 329
Pregnancy
  circulating parathyroid hormone in, 228, 229
  evaluation of duration of, 165-67
  hormonal imbalance in
    and heart disease, 62
  and myocarditis, 149
  prolactin levels in, 257, 258, 264
  use of methadone in, 162
Premature infants
  see Infants, premature
Probenecid
  drug interaction of, 389
  in Lesch-Nyhan treatment, 57
Procainamide
  drug interactions with, 400, 401
Procarbazine
  in treatment of Hodgkin's disease, 327, 328
Progesterone

  - neonatal metabolism of, 30
  - on platelet function, 11
- Proinsulin
  - binding of, 234
- Prolactin, human (hPRL), 251-70
  - amino acid sequence, 254
  - assays for, 255, 256
  - and cancer, 265, 266
  - causes of elevated levels of, 264
  - cell identification, 253
  - control of secretion, 259-65
  - physiological role, 266, 267
  - purification and chemistry, 253-55
  - separation from growth hormone, 251, 252
  - serum concentrations in different clinical states, 256-59
  - summary, 267, 268
  - and TRH, 357, 359, 360
- Prolactin-inhibiting factor (PIF)
  - source of, 259, 261
- Prolactin-releasing factor (PRF)
  - evidence for, 259
- Prolactin, sheep
  - amino acid sequence of, 254
- Promazine
  - neonatal effects of, 34
- Propranolol
  - bronchospasm from, 130
  - drug interactions with, 402, 403, 407
- Prostaglandins
  - on intestinal fluid production, 20, 26
  - on TRH secretion, 358
- Prostate
  - cancer of
    - treatment of, 260
- Prostatic secretions
  - antibacterial, 85
- Protamine
  - drug interactions of, 385
- Protriptyline
  - drug interactions with, 410
- Pseudohypoparathyroidism
  - circulating PTH in, 224
- Pseudomonas aeruginosa
  - toxins in, 107
- Psoriasis
  - cytokinetics of, 346-49
  - treatment of, 350
- Psychosis
  - following L-dopa, 210-14
- Psychotropics
  - drug interactions with, 407-13
    - amphetamines, 409
    - antagonism of guanidinium antihypertensives, 410, 411
    - interactions at adrenergic neuron, 410
    - monoamine oxidase inhibitors, 409
    - phenothiazines, 407, 408
    - treatment of depressed cardiac patient, 411, 412
    - tricyclic antidepressants, 408, 409
    - useful interactions of adrenergic drugs, 412, 413
  - on prolactin secretion, 259, 264
- Puberty
  - prolactin levels at, 257
- Pulmonary stenosis
  - valvar type
    - genetics of, 63
- Purine
  - metabolism of
    - disorders of, 54-56
    - in Lesch-Nyhan syndrome, 45, 46
- Puromycin
  - infant metabolism of, 31
- Pyridinolcarbamate
  - on platelet function, 10
- Pyrogen
  - on TSH secretion, 356
- L-Pyroglutamyl-L-histidyl-L-proline amide
  - see Thyrotropin releasing hormone
- Pyruvate
  - on cerebral glucose utilization, 273
  - renal utilization of, 369

Q

- Quinacrine fluorescence (Q-banding)
  - in chromosome identification, 67-69
    - photographs of, 69
- Quinidine
  - drug interactions with, 396, 400
- Quinine
  - drug interactions of, 396

R

- Radiation
  - as cause of leukemia, 75-82
  - immunodeficiency from, 135, 140
- Radiation sickness
  - and resistance to infection, 293
- Radioimmunoassay
  - of gonadotropic hormones, 295, 298-30
  - for human prolactin, 254-56
  - of parathyroid hormone, 220-23
    - immunochemical heterogeneity of circulating PTH, 221, 222
    - problems in interpretation, 222, 223
    - technical aspects, 220, 221
  - for TSH, 358, 360
- Radioreceptor assay
  - of prolactin, 255, 256
- Radiotherapy
  - in Hodgkin's disease, 326, 327
    - combination with drug therapy, 329-31
    - complications of, 327
  - in lymphoma, 332
- R-banding
  - in chromosome identification, 72
- Receptors
  - drug interactions at, 388
  - insulin, 233-40
- Red blood corpuscle
  - and carbon monoxide, 247
- Reptilase
  - clotting role of, 336
- Reserpine
  - and L-dopa, 212
  - on prolactin levels, 264
- Respiration
  - mechanics of airway obstruction, 111-34
  - normal airway function, 112-19
    - distribution of ventilation, 117-19
    - expiration, 115-17
    - extrathoracic airways, 113, 114
    - inspiration, 114, 115
    - intrathoracic airways, 114-19
- Reticular agenesis
  - immune deficiency in, 185
- Reticulocytosis, ineffective
  - and $V_{CO}$, 247
- Rheumatoid arthritis
  - ascorbic acid levels in, 392
- Rickets
  - parathyroid hormone levels in, 228
- Rosette-forming cells (RFC)
  - in immune response, 182
- Rubella
  - cardiac effects of, 146, 149

S

Salbutamol
  in bronchospasm, 129
Salicylates
  interactions of, 391-93, 413, 414
Salicylic acid
  drug interactions with, 391
Sarcoidosis
  circulating parathyroid hormone in, 229
  immune deficiency in, 187
  prolactin levels in, 264
  treatment of, 198, 199
Sarcoma
  hCG in, 299
  immune deficiency in, 187, 202
    transfer factor therapy, 202
Schizophrenia
  behavioral effects of L-dopa in, 211, 213, 214
  childhood
    platelet function in, 13
Scurvy
  platelet function in, 13
Sebum
  in amniotic fluid, 167
Self-mutilation
  in Lesch-Nyhan syndrome, 42
Semilunar valve anomalies
  genetics of, 63, 64
Senile emphysema
  obsolescence of term, 126
Septicemia
  platelet function in, 5
Serotonin
  and L-dopa, 213
  and platelet function, 2, 6, 8, 10, 13
Serum prothrombin time
  in platelet function tests, 3
Sex chromosomes
  see Chromosomes, sex
Shigella dysenteriae
  enterotoxins in, 107
Shock
  platelet function in, 6
Sialic acid
  in gonadotropic hormones, 295, 296
    desialylation, 296
Skin
  cytokinetics of epidermal hyperplasia, 345-52
Skin-reactive factors
  in immune response, 178, 179
Smokers
  airway obstruction in, 111, 112, 122, 126, 127, 130
Smooth muscle
  effect of fibrinogen degradation products on, 338, 339
Snake venoms
  clotting action of, 336
Sodium
  effect of cholera toxin on movement of, 22
  renal absorption and energy metabolism, 365-84
Sodium chloride
  on insulin binding, 238
Sodium dodecyl sulfate (SDS)-urea polyacrylamide gel electrophoresis
  in study of fibrinogen-fibrin degradation products, 337
Sodium-potassium adenosine triphosphatase (Na, K-ATPase)
  on intestinal fluid and electrolyte movement
    cholera toxin effects on, 24-27
  in kidney, 375, 378-81
Somatomammotropin
  in estimation of gestational age, 166, 167
Sphingomyelin
  in estimation of fetal age, 169
Spironolactone
  drug interactions with, 406
Splenectomy
  in chronic myelocytic leukemia, 284
  in treatment of Hodgkin's disease, 325
Starvation
  host resistance to infection in, 293
  insulin:glucagon ratios in, 304, 305
  ketone body utilization by brain in, 273-75, 277
Streptokinase-Streptodornase antigen
  in assessment of immunity, 180, 202
Streptomycin
  preventive use in surgery, 289, 291
Streptozotocin
  on insulin receptors, 237
Stress
  and prolactin levels, 259, 264
  on TSH secretion, 357
Stroke
  platelet function in, 9, 10
Succinate
  on cerebral glucose utilization, 272
Succinylcholine
  neonatal metabolism of, 35
Sulfadimethoxine
  drug interactions with, 404
Sulfamethazine
  drug interactions with, 390
Sulfinpyrazone
  drug interaction of, 389, 392, 396
Sulfobromophthalein
  infant metabolism of, 34
Sulfonamides
  drug interactions with, 396
Supravalval anomalies
  genetics of, 64
Surgery
  preventive antibiotic management in, 289-94
    classification of patients, 293
    host-defense mechanisms, 290, 291
    statement of concept of, 292
Surgical operations
  platelet function tests after, 5, 6
Syndromes
  Bernard-Soulier giant platelet
    platelet function tests in, 4
  Bloom's
    and leukemia, 80
  Chediak-Higashi
    site of defect in, 185, 187
    therapy of, 197
  Chiari-Frommel
    prolactin levels in, 264
  DiGeorge
    genetics of, 62
    site of defect in, 185, 187, 190
    therapy of, 190
    thymus transplant in, 141
  Down's
    androgen therapy of, 98, 99
    behavioral genetics of, 93-95, 98
    chromosome identification in, 67, 68, 70
    comparison of home-reared and institutionalized, 94
    drug interactions in, 392
    heart defects with, 62, 63
    platelet function in, 13
  Ellis-van Creveld
    genetics of, 63
  enterotoxic diarrheal, 103-10
  Fanconi's anemia
    and leukemia, 80
  Grey, of infants

    drug metabolism role in, 35
  hypercalcemia
    genetics of, 64
  Job's
    site of defect in, 185, 187
    therapy of, 196, 197
  Kasabach-Merritt
    platelet function in, 7
  Klinefelter's
    behavioral aspects of, 90-93, 96, 99
  Lesch-Nyhan, 41-60
    amniocentesis-intrauterine diagnosis, 51, 52
    clinical features, 41-44
    genetics, 49-51
    metabolic features, 45, 46
    molecular defect, 47-49
    other disorders of purine metabolism-molecular heterogeneity, 54-56
    pathogenesis of cerebral manifestations, 53, 54
    somatic cell genetics and biology, 52, 53
    treatment, 56-58
  lupus erythematosus-like
    drug-induced, 401, 407
  Marfan's
    genetics of, 64
  Nelson's
    prolactin levels in, 264
  neonatal respiratory distress
    prevention of, 168, 169
  Nezeloff
    site of defect in, 185, 187, 190, 191
    therapy of, 191
  Noonan's
    genetics of, 63, 65
  thrombocytopenia with absent radius
    genetics of, 62
  Turner's
    behavioral aspects of, 89-91, 97, 99
    gender orientation, 90
    genetics of, 64
  Wiskott-Aldrich
    immunologic reconstitution in, 140, 142
    platelet function tests in, 4
    site of defect, 185, 187
    transfer factor therapy in, 175, 183, 193-96
  withdrawal from drugs
    treatment of, 157
Syphilis
  platelet function in, 5

T

T-cell defects
  characterization of, 186-90
  site of defect in, 185
Testes
  prolactin effects in, 267
Testicular neoplasia
  hCG in, 299
Tetrachlorosalicylanilide
  to study cytokin tics, 347
Tetracycline
  in cholera, 105
Tetralogy of Fallot
  familial aggregations in, 62
Textile dusts
  as cause of airway obstruction, 111
Theophylline
  on intestinal fluid production, 20
  to simulate Lesch-Nyhan syndrome, 54
  in treatment of bronchospasm, 129
  on TRH secretion, 358
6-Thioguanine
  in Lesch-Nyhan syndrome, 51
  in leukemia chemotherapy, 285
Thrombasthenia
  platelet function tests in 4
Thrombin
  clotting by, 336
  and platelet aggregation, 2, 4
Thrombocytopenia
  platelet function in, 4, 5, 7
Thrombosis
  diagnosis of, 341
  platelet function in, 5, 9-11
Thymic alymphoplasia
  characterization and treatment of, 191, 192
Thymic aplasia
  immune deficiency in, 190
Thymic epithelial arrests
  site of defect in, 185, 187
Thymidine labeling
  to study skin hyperplasis, 346, 347, 349
Thymus
  transplantation of, 141, 142
Thyroid cancer
  leukemogenesis by therapy in, 76
Thyroid hormones
  antagonism of TRH by, 358
  drug interactions with, 392
Thyrotropin releasing hormone (TRH), 253-64
  biosynthesis of, 354, 355
  degradation of, 355, 356
  introduction, 353
  in man, 358-62
    euthyroid subjects with pituitary tumors, 362
    in hyperthyroid states, 361, 362
    in hypothyroid states, 360, 361
    pharmacological modifications of TRH stimulation in man, 362
    secondary TRH influences on thyroid physiology, 360
    specificity of TRH activity in normal man, 359, 360
    summary of clinical applications, 362
    TSH release after TRH in normals, 358, 359
    untoward effects, 360
  mechanism of action of, 357, 358
  pituitary receptor purified, 357
  on prolactin secretion, 259-65
    mechanism of action, 262
  secretory physiology, 356, 357
  structure-activity relationships, 353, 354
Tolbutamide
  drug interactions with, 404
  neonatal metabolism of, 35
Toluene diisocyanate vapor
  as cause of airway obstruction, 111, 127
Toxemia
  amniotic fluid creatinine levels in, 168
Trachea
  airways of, 113
Tracheomalacia
  airway obstruction by, 120
Transfer factor
  assessment of cellular immunity, 179-84
    antigens, table of, 180
    methods, 180
    schematic of assay for lymphocyte transformation, 181
    schematic of assay for Macrophage Migration Inhibition Assay (MIF), 182
    typical MIF patterns, 183
  chemical character of, 175-77

  in immune deficiency therapy, 175-208
    classification of states and response, 186-204
    conclusion, 204
    development of immune response, 184-86
    introduction, 184
  in immunologic reconstitution, 142
  mode of action of, 177-79
    antiviral and other factors, 179
    factors acting primarily on other lymphocytes, 178
    factors acting primarily on macrophages, 178, 179
    necrotizing factor, 179
    specificity of, 177
Trauma
  alpha cell function in, 306
Trichloroacetic acid
  drug interactions with, 396
Trichophytin
  in assessment of immunity, 180
Tricyclic antidepressants
  and L-dopa, 212, 213
  drug interactions with, 408, 409, 411, 412, 414
L-Triiodothyronine ($T_3$)
  on TRH activity, 356, 358
Triton X-100
  on insulin receptor, 238
Trypsin
  clotting action of, 336
  on insulin receptor, 236
L-Tryptophan
  on prolactin secretion, 260
Tuberculosis
  immune deficiency in, 187
    treatment, 198, 200
  and resistance to infections, 293
Tyramine
  drug interactions with, 410

U

Ultrasound
  correlation with caliper measurements, 170
  in estimation of gestational age, 169-71
  in location of placenta, 169
  safety of, 171
  ultrasonogram of fetal heart, 170
Uracil mustard
  in chronic myelocytic leukemia, 283, 284
Urates
  drug interactions of, 392
Uremia
  drug interactions in, 387
  platelet function in, 7, 8
Urethane
  leukemogenesis studies, 78
Urethrovesical dysfunction and infection, 83-88
  factors limiting perineal and urethral colonization, 84, 85
  hydrodynamic mechanisms of, 85, 86
  source of pathogenic bacteria, 83, 84
  summary, 87
Uric acid
  in Lesch-Nyhan syndrome, 44, 45
Urinary infection
  causes of, 83-88
Ussing chamber
  to study cholera, 104

V

Vagina
  cytokinetics of, 348
Varicella myocarditis
  arrhythmias from, 146
Venous thrombosis
  platelet function tests of, 5
Ventilation
  distribution of, 117-19, 125, 126
    normal, 117-19
    testing of, 125, 126
Ventricular septal defect
  genetics of, 63
Vibrio cholerae
  diarrheal syndrome, 103-5, 107
  on intestinal fluid and ion movement, 19-27
Vinblastine
  in treatment of Hodgkin's disease, 327-29, 331
  in treatment of skin hyperplasia, 350
Vincristine
  on platelet function, 12
  in treatment of Hodgkin's disease, 327-29
  in treatment of lymphomas, 332
Viral myocarditis and sequelae, 145-52
  cardiac arrhythmias and conduction disturbances, 146
  chronic, 147-49
  clinical aspects, 145
  concluding remarks, 150
  incidence of, 145
  miscellaneous experimental studies, 149
  sudden death, 146, 147
Virus infections
  as cause of airway obstruction, 112
  platelet function tests in, 5
  transfer factor treatment of, 200, 201
Visceroatrial situs
  congenital anomalies of, 62
Vitamin $B_{12}$
  drug interactions with, 390
Vitamin D
  and circulating PTH, 228
  drug interactions with, 399
Vitamin K
  drug interactions with, 393, 394

W

Warfarin
  drug interactions of, 388, 390, 393, 394, 396, 404
Water
  cholera toxin effects on movement of, 19-21
Wheat germ agglutinin
  insulin-like properties of, 237, 238

X

Xanthine oxidase
  drug interaction with, 389
X-ray
  leukemogenesis by, 76, 77
  and platelet function, 3
D-Xylose
  drug interactions with, 392

# CUMULATIVE INDEXES

## VOLUMES 20-24

## INDEX OF CONTRIBUTING AUTHORS

A

Abelmann, W. H., 24:145
Ardaillou, N., 20:47
Armitage, P., 20:425

B

Bagdade, J. D., 21:219
Baldessarini, R. J., 23:343
Barlow, G. H., 24:335
Barnes, A. C., 22:133
Bausher, J. C., 23:39
Becker, F. F., 20:243
Bellingham, A. J., 22:221
Benacerraf, B., 20:141
Bertino, J. R., 21:433
Bidwell, E., 20:63
Biggar, W. D., 24:135
Bitensky, M. W., 23:263
Blumberg, B. S., 23:161
Bodey, G. P., 21:105
Bothwell, T. H., 21:145
Bouhuys, A., 24:111
Boyarsky, S., 20:383
Brady, R. O., 21:317
Brain, M. C., 21:133
Breslow, J. L., 24:315
Brusilow, S. W., 21:99
Burch, G. E., 22:327
Burchenal, J. H., 23:77
Burke, J. F., 24:289

C

Canterbury, J. M., 24:217
Capizzi, R. L., 21:433
Carpenter, C. B., 21:1
Charlton, R. W., 21:145
Childs, B., 23:373
Coburn, R. F., 24:241
Cohn, K. E., 21:47
Coombs, J., 23:149
Cooperband, S. R., 22:185
Cotzias, G. C., 22:305
Creger, W. P., 24:75
Cuatrecasas, P., 24:233

D

Dao, T. L., 23:1
De Clercq, E., 21:17
DeGraff, A. C., 24:111
DePasquale, N. P., 22:327
Desbuquois, B., 24:217
Dowling, M. D., Jr., 23:77
Dreizen, P., 22:365
Dresch, C., 20:47
Dripps, R. D., 22:293
Düby, S., 22:305
DuPont, H. L., 24:103
Dupré, J., 21:299

E

Edelmann, C. M., Jr., 20:363
Eriksson, M., 24:29

F

Fanestil, D. D., 20:223
Farber, E. M., 21:59
Field, J. B., 24:309
Finch, C. A., 22:221
Fomon, J. J., 20:277
Formal, S. B., 24:103
Fox, A. C., 22:355
Frangione, B., 20:155
Franklin, E. C., 20:155
Fredrickson, D. S., 24:315
Freedman, D. X., 20:405; 24:153
Friesen, H. G., 24:251
Fudenberg, H. H., 22:145; 24:175

G

Gershon, S., 23:439
Gill, J. R., Jr., 21:269
Ginos, J., 22:305
Gitelman, H. J., 20:233
Glassman, E., 23:229
Gold, P., 22:85
Goldstein, G., 22:119
Good, R. A., 24:135
Gorman, R. E., 23:263
Gottschalk, A., 20:131
Gray, G. M., 22:391
Green, I., 20:141
Guenter, C. A., 22:283

H

Hammarsten, J. F., 22:283
Handschumacher, R. E., 21:433
Hatch, F. E., 20:395
Havens, L. L., 20:419
Hellström, I., 23:19
Hellström, K. E., 23:19
Henkind, P., 22:95
Hersh, E. M., 21:105
Hinman, F., 24:83
Hirschhorn, K., 24:67
Hoffman, J. I. E., 20:15
Hofmann, W. W., 20:101
Holtzman, N. A., 21:335
Hornick, R., 24:103
Horton, E. S., 22:235
Hossfeld, D. K., 21:379
Huehns, E. R., 21:157
Hurvitz, A. I., 23:191
Hwang, P., 24:251

I

Ishizaka, K., 21:187
Israel, C. W., 22:103

J

Jacob, H. S., 20:41
Janoff, A., 23:177
Johnson, J. C., 20:315
Johnson, J. G., 20:395
Johnson, J. W. C., 24:165

K

Kalkhoff, R. K., 23:429
Kaltman, A. J., 22:343
Karpatkin, S., 23:101
Kayden, H. J., 23:285
Keele, C. A., 21:67
Kellogg, D. S., Jr., 20:323
Kessler, R. H., 23:365
Kessler, S., 24:89
King, L. J., 21:367
Klebanoff, S. J., 22:39
Kleeman, C. R., 21:259
Klion, F. M., 19:25
Koenig, M. G., 22:25
Kolff, W. J., 23:321
Kwaan, H. C., 24:335
Kyle, L. H., 20:259

L

Labay, P., 20:383
La Du, B. N., 23:453
Lane, J. M., 22:251
Lawrence, A. M., 20:207

Leader, R. W., 23:191
Legator, M. S., 23:413
Lenfant, C., 22:221
Levin, A. S., 24:175
Levin, N. W., 24:365
Lewy, P. R., 24:365
Littlefield, J. W., 23:57
London, W. T., 23:161
Lowenstein, J., 23:333
Luby, J. P., 20:329

M

McCarty, D. J., 21:357
McCullough, J. L., 24:345
McGee, Z. A., 22:25
Menguy, R., 23:297; 313
Merigan, T. C., 21:17
Millar, J. D., 22:251
Millman, I., 23:161
Mills, I. H., 21:75
Mills, J. A., 22:185
Milunsky, A., 23:57
Moos, R. H., 24:89
Morse, S. I., 20:357
Moss, G. S., 23:201
Murphy, D. L., 24:209

N

Najean, Y., 20:47
Neff, J. M., 22:251
Neill, C. A., 24:61
Norton, W. L., 20:351
Nyhan, W. L., 24:41

O

Odell, G. B., 24:165

P

Papavasiliou, P. S., 22: 305
Park, B. H., 24:135
Paterson, P. Y., 20:75
Perkoff, G. T., 22:125
Perry, S., 22:171
Pierce, A. K., 20:431
Pink, R., 22:145
Porte, D., Jr., 21:219
Potolsky, A., 24:75

Q

Quintanilla, A., 24:365

R

Rabinowitz, D., 21:241
Rabinowitz, M., 24:245
Raisfeld, I. H., 24:385
Ramot, B., 22:19
Reiss, E., 24:217
Remington, J. S., 21:201
Rodriguez-Soriano, J., 20: 363
Rosenberg, E. W., 20: 201
Rosomoff, H. L., 20:189
Ross, G. T., 24:295
Rubenstein, A. H., 22:1

S

Salans, L. B., 22:235
Sandberg, A. A., 21: 379
Sanford, J. P., 20:329
Schaffner, W., 22:25
Seligman, M. E. P., 23:407
Selzer, A., 21:47
Senay, E. C., 24:153
Sharp, G. W. G., 24:19
Shaw, M. W., 21:409
Sims, E. A. H., 22:235
Sinclair, J. C., 23:129
Singal, D. P., 20:175
Smith, J. L., 22:103
Smith, R. T., 23:39
Sokal, J. E., 24:281
Sokoloff, L., 24:271
Spencer, F. C., 23:229
Spitler, L., 24:175
Stamatoyannopoulos, G., 22:221
Stead, E. A., Jr., 22:273
Steck, A., 22:305
Steiner, D. F., 22:1
Stollerman, G. H., 20: 315
Strandness, D. E., Jr., 20:1
Stutzman, L., 24:325
Sulkin, S. E., 20:329
Sutnick, A. I., 23:161

T

Tan, C. T. C., 23:77
Tantum, K. R., 22:293
Terasaki, P. I., 20:175
Thayer, J. D., 20:323
Thomas, L., 21:179
Thompson, J. C., 20:291
Tomasi, T. B., Jr., 21:281
Tschopp, T. B., 24:1

U

Unger, R. H., 24:303

V

Vaitukaitis, J., 23:295
Valentine, W. N., 23:93

W

Wang, A.-C., 22:145
Warren, W. D., 20:277
Weinstein, G. D., 24:345
Weiss, L., 20:29
Welch, M. H., 22:283
Welt, L. G., 20:233
Wender, P. H., 23:355
Wennberg, E., 20:29
Wilber, J. F., 24:353
Wilhelm, D. L., 22:63
Winick, M., 23:149

Y

Yaffe, S. J., 25:29

Z

Zackheim, H. S., 21:59
Zak, R., 24:245
Zucker, M. B., 24:1

# INDEX OF CHAPTER TITLES

## VOLUMES 20-24

| | | |
|---|---|---|
| ANESTHESIA | | |
| Neurosurgical Control of Pain | H. L. Rosomoff | 20:189-200 |
| CARDIOLOGY | | |
| Pathological Alterations in Blood Flow | D. E. Strandness, Jr. | 20:1-14 |
| The Natural History of Congenital Isolated Pulmonic and Aortic Stenosis | J. I. E. Hoffman | 20:15-28 |
| Treatment of Ventricular Extrasystoles and Tachyarrhythmias in Acute Myocardial Infarction | A. Selzer, E. E. Cohn | 21:47-58 |
| Papillary Muscle Dysfunction in Coronary (Ischemic) Heart Disease | N. P. DePasquale, G. E. Burch | 22:327-42 |
| Late Complications of Heart Valve Replacement | A. J. Kaltman | 22:343-54 |
| Abnormalities in Cardiac Contractility Associated with Localized Myocardial Damage | A. C. Fox | 22:355-64 |
| Structure and Function of the Myofibrillar Contractile Proteins | P. Dreizen | 22:365-90 |
| Pulmonary Involvement in Hypovolemic Shock | G. S. Moss | 23:201-28 |
| Surgical Procedures for Coronary Artery Disease | F. C. Spencer, E. Glassman | 23:229-44 |
| Biochemical and Cellular Changes in Cardiac Hypertrophy | M. Rabinowitz, R. Zak | 23:245-62 |
| Genetics of Congenital Heart Disease | C. A. Neill | 24:61-67 |
| Viral Myocarditis and Its Sequelae | W. H. Abelmann | 24:145-52 |
| Primary Hyperlipoproteinemia in Infants | D. S. Fredrickson, J. L. Breslow | 24:315-24 |
| CLINICAL PHARMACOLOGY | | |
| Metabolic Modification of Parkinson's Disease and of Chronic Manganese Poisoning | G. C. Cotzias, P. S. Papavasiliou, J. Ginos, A. Steck, S. Duby | 22:305-26 |
| Effects of Oral Contraceptive Agents and Sex Steroids on Carbohydrate Metabolism | R. K. Kalkhoff | 23:429-38 |
| Lithium Salts in the Management of the Manic-Depressive Syndrome | S. Gershon | 23:439-52 |
| Pharmacogenetics: Defective Enzymes in Relation to Reactions to Drugs | B. N. La Du | 23:453-68 |
| Drug Metabolism in the Newborn | M. Eriksson, S. J. Yaffe | 24:29-40 |
| Clinical Pharmacology of Drug Interactions | I. H. Raisfeld | 24:385-418 |
| DERMATOLOGY | | |
| Bacteriology of Acne | E. W. Rosenberg | 20:201-6 |
| Topical Antimetabolites | H. S. Zackheim, E. M. Farber | 21:59-66 |
| Chemical Causes of Pain and Itch | C. A. Keele | 21:67-74 |
| Cytokinetics in Diseases of Epidermal Hyperplasia | G. D. Weinstein, J. L. McCullough | 24:345-52 |
| ENDOCRINOLOGY | | |
| Glucagon | A. M. Lawrence | 20:207-22 |
| Mechanism of Action of Aldosterone | D. D. Fanestil | 20:223-32 |
| Magnesium Deficiency | H. J. Gitelman, L. G. Welt | 20:233-42 |
| Cell Division in Normal Mammalian Tissues | F. F. Becker | 20:243-58 |
| Glomerular (Azotemic) Osteodystrophy | L. H. Kyle | 20:259-76 |
| Human Insulin Secretion: An Integrated Approach | D. Porter Jr., J. D. Bagdade | 21:219-40 |
| Some Endocrine and Metabolic Aspects of Obesity | D. Rabinowitz | 21:241-58 |
| Hypo-Osmolar Syndromes Secondary to Impaired Water Excretion | C. R. Kleeman | 21:259-68 |
| Edema | J. R. Gill Jr. | 21:269-80 |

| | | |
|---|---|---|
| Proinsulin | A. H. Rubenstein, D. F. Steiner | 22:1-18 |
| Chemical Mediation of Hormone Action | M. W. Bitensky, R. E. Gorman | 23:263-84 |
| Abetalipoproteinemia | H. J. Kayden | 23:285-96 |
| Blood Levels of Parathyroid Hormone in Disorders of Calcium Metabolism | E. Reiss, J. M. Canterbury | 24:217-32 |
| Insulin Receptors | B. Desbuquois, P. Cuatrecasas | 24:233-40 |
| Human Prolactin | H. Friesen, P. Hwang | 24:251-70 |
| Recent Advances in Evaluation of Gonadotropic Hormones | J. L. Vaitukaitis, G. T. Ross | 24:295-302 |
| Normal and Abnormal Alpha Cell Function | R. H. Unger | 24:303-8 |
| Thyrotropin Releasing Hormone: Secretion and Actions | J. F. Wilber | 24:353-64 |

GASTROENTEROLOGY

| | | |
|---|---|---|
| Hemodynamic Studies in Portal Hypertension | J. J. Fomon, W. D. Warren | 20:277-90 |
| Gastrin and Gastric Secretion | J. C. Thompson | 20:291-314 |
| Structure and Function of Mucosal Antibodies | T. B. Tomasi Jr. | 21:281-98 |
| Regulation of the Secretions of the Pancreas | J. Dupré | 21:299-316 |
| Malabsorption Due to Lymphomatous Disease | B. Ramot | 22:19-24 |
| Intestinal Digestion and Maldigestion of Dietary Carbohydrates | G. M. Gray | 22:391-404 |
| Acute Gastric Mucosal Bleeding | R. Menguy | 23:297-312 |
| Choice of Operations in the Management of Symptomatic Sliding Hiatal Hernia | R. Menguy | 23:313-20 |
| Action of Cholera Toxin on Fluid and Electrolyte Movement in the Small Intestine | G. W. G. Sharp | 24:19-28 |
| Enterotoxic Diarrheal Syndromes | S. B. Formal, H. L. DuPont, R. B. Hornick | 24:103-10 |
| Extraction of Insulin by Liver | J. B. Field | 24:309-14 |

HEMATOLOGY

| | | |
|---|---|---|
| The Structure of the Spleen and Hemolysis | E. Wennberg, L. Weiss | 20:29-40 |
| The Defective Red Blood Cell in Hereditary Spherocytosis | H. S. Jacob | 20:41-46 |
| Platelet Lifespan | Y. Najean, N. Ardaillou, C. Dresch | 20:47-62 |
| Acquired Inhibitors of Coagulants | E. Bidwell | 20:63-74 |
| Leukocytic Mechanisms in Inflammation | E. M. Hersh, G. P. Bodey | 21:105-32 |
| Microangiopathic Hemolytic Anemia | M. C. Brain | 21:133-44 |
| Absorption of Iron | T. H. Bothwell, R. W. Charlton | 21:145-56 |
| Diseases Due to Abnormalities of Hemoglobin Structure | E. R. Huehns | 21:157-78 |
| Proliferation of Myeloid Cells | S. Perry | 22:171-84 |
| Lymphocyte Physiology | J. A. Mills, S. R. Cooperband | 22:185-220 |
| Abnormal Hemoglobins with High and Low Oxygen Affinity | G. Stamatoyannopoulos, A. J. Bellingham, C. Lenfant, C. A. Finch | 22:221-34 |
| Treatment of Acute Lymphoblastic Leukemia | J. H. Burchenal, M. D. Dowling Jr., C. T. C. Tan | 23:77-92 |
| Red Cell Enzyme Deficiencies as a Cause of Hemolytic Disorders | W. N. Valentine | 23:93-100 |
| Human Platelet Senescence | S. Karpatkin | 23:101-28 |
| Platelet Function Tests in Disease | T. B. Tschopp, M. B. Zucker | 24:1-18 |
| Radiation and Drug Therapies, and Leukemia | A. Potolsky, W. P. Creger | 24:75-82 |
| Current Concepts in the Treatment of Chronic Myelocytic Leukemia | J. E. Sokal | 24:281-88 |
| Nature and Biological Activities of Degradation Products of Fibrinogen and Fibrin | H. C. Kwaan, G. H. Barlow | 24:335-44 |

IMMUNOLOGY

| | | |
|---|---|---|
| Immune Processes and Infectious Factors | | |

| | | |
|---|---|---|
| in Central Nervous System Disease | P. Y. Paterson | 20:75-100 |
| Cellular Hypersensitivity | B. Benacerraf, I. Green | 20:141-54 |
| Immunoglobulins | E. C. Franklin, B. Frangione | 20:155-74 |
| Human Histocompatibility Antigens of Leukocytes | P. I. Terasaki, D. P. Singal | 20:175-88 |
| Immunologic Aspects of Renal Disease | C. B. Carpenter | 21:1-16 |
| Current Concepts of Interferon and Interferon Induction | E. De Clercq, T. C. Merigan | 21:17-46 |
| Antibody Variability | R. Pink, A.-C. Wang, H. H. Fudenberg | 22:145-70 |
| Neutrophil Proteases in Inflammation | A. Janoff | 23:177-90 |
| Interspecies Patterns of Slow Virus Diseases | R. W. Leader, A. I. Hurvitz | 23:191-200 |
| Immunologic Reconstitution | W. D. Biggar, B. H. Park, R. A. Good | 24:135-44 |
| Transfer Factor Therapy in Immune Deficiency States | A. S. Levin, L. E. Spitler, H. H. Fudenberg | 24:175-208 |
| INFECTIOUS DISEASE | | |
| Nephritogenic Streptococci | J. C. Johnson, G. H. Stollerman | 20:315-22 |
| Virulence of Gonococci | D. S. Kellogg Jr., J. D. Thayer | 20:323-28 |
| The Epidemiology of St. Louis Encephalitis: A Review | J. P. Luby, S. E. Sulkin, J. P. Sanford | 20:329-50 |
| Chlamydial Infection in Reiter's Syndrome | W. L. Norton | 20:351-56 |
| The Role of Bacteriophages in Bacterial Resistance to Antimicrobials | S. I. Morse | 20:357-62 |
| Mycoplasmas as Infectious Agents | L. Thomas | 21:179-86 |
| Human Reaginic Antibodies | K. Ishizaka | 21:187-200 |
| Toxoplasmosis: Recent Developments | J. S. Remington | 21:201-18 |
| Superinfection in Lymphoreticular Diseases | Z. A. McGee, W. Schaffner, M. G. Koenig | 22:25-38 |
| The Role of Australia Antigen in Viral Hepatitis and Other Diseases | A. I. Sutnick, I. Millman, W. T. London, B. S. Blumberg | 23:161-76 |
| Preventive Antibiotic Management in Surgery | J. F. Burke | 24:289-94 |
| MISCELLANY | | |
| Human Chromosome Damage by Chemical Agents | M. W. Shaw | 21:409-32 |
| Kinins in Human Disease | D. L. Wilhelm | 22:63-84 |
| Chromosome Identification | K. Hirschhorn | 24:67-74 |
| MUSCULO-SKELETAL DISORDERS | | |
| Membrane Potential and Muscle Function | W. W. Hofmann | 20:101-30 |
| Crystal-Induced Inflammation of the Joints | D. J. McCarty | 21:357-66 |
| Myasthenia Gravis and the Thymus | G. Goldstein | 22:119-24 |
| Alcoholic Myopathy | G. T. Perkoff | 22:125-32 |
| NEPHROLOGY | | |
| Renal Tubular Acidosis | J. Rodriguez-Soriano, C. M. Edelmann Jr. | 20:363-82 |
| Ureteral Motility | S. Boyarsky, P. Labay | 20:383-94 |
| Intrarenal Blood Flow | F. E. Hatch, J. G. Johnson | 20:395-408 |
| Renal Regulation of Sodium Excretion | I. H. Mills | 21:75-98 |
| Intraleukocytic Microbicidal Defects | S. J. Klebanoff | 22:39-62 |
| Hemodialysis in the Management of Renal Disease | W. J. Kolff | 23:321-32 |
| Renin Assay in Hypertensive Disease | J. Lowenstein | 23:333-42 |
| Urethrovesical Dysfunction and Infection | F. Hinman | 24:83-88 |
| Renal Energy Metabolism and Sodium Reabsorption | P. R. Lewy, A. Quintannila, N. W. Levin, R. H. Kessler | 24:365-84 |
| NEUROLOGY | | |
| Cerebral Lipidoses | R. O. Brady | 21:317-34 |
| Thermal Control in Premature Infants | J. C. Sinclair | 23:129-48 |
| Nutrition, Environment, and Behavioral Development | M. Winick, J. Coombs | 23:149-60 |

| | | |
|---|---|---|
| The Lesch-Nyhan Syndrome | W. L. Nyhan | 24:41-60 |
| Metabolism of Ketone Bodies by the Brain | L. Sokoloff | 24:271-80 |
| NUTRITION | | |
| Dietary Treatment of Inborn Errors of Metabolism | N. A. Holtzman | 21:335-56 |
| Inducible Metabolic Abnormalities During Development of Obesity | E. A. H. Sims, E. S. Horton, L. S. Salans | 22:235-50 |
| ONCOLOGY | | |
| Chromosomal Abnormalities in Human Neoplasia | A. A. Sandberg, D. K. Hossfeld | 21:379-408 |
| L-Asparaginase | R. L. Capizzi, J. R. Bertino, R. E. Handschumacher | 21:433-44 |
| Antigenic Reversion in Human Cancer | P. Gold | 22:85-94 |
| Ablation Therapy for Hormone-Dependent Tumors | T. L. Dao | 23:1-18 |
| Immunity to Neuroblastomas and Melanomas | K. E. Hellström, I. Hellström | 23:19-38 |
| Epstein-Barr Virus Infection in Relation to Infectious Mononucleosis and Burkitt's Lymphoma | R. T. Smith, J. C. Bausher | 23:39-56 |
| Current Concepts in Treatment of Lymphomas with Special Reference to Hodgkin's Disease | L. Stutzman | 24:325-34 |
| OPHTHALMOLOGY AND OTORHINOLARYNGOLOGY | | |
| Senile Macular Degeneration | P. Henkind | 22:95-102 |
| Optic Atrophy and Neurosyphilis | J. L. Smith, C. W. Israel | 22:103-18 |
| PSYCHIATRY | | |
| The Psychopharmacology of Hallucinogenic Agents | D. X. Freedman | 20:409-18 |
| Diagnosis of Suicidal Intent | L. L. Havens | 20:419-24 |
| Chemotherapy of Mental Depression | L. J. King | 21:367-78 |
| Biogenic Amines and Behavior | R. J. Baldessarini | 23:343-54 |
| Adopted Children and Their Families in the Evaluation of Nature-Nurture Interactions in the Schizophrenic Disorders | P. H. Wender | 23:355-72 |
| Genetic Analysis of Human Behavior | B. Childs | 23:373-406 |
| Learned Helplessness | M. E. P. Seligman | 23:407-12 |
| Chemical Mutagens | M. S. Legator | 23:413-28 |
| Behavioral Aspects of Chromosomal Disorders | S. Kessler, R. H. Moos | 24:89-102 |
| Mental Effects of L-Dopa | D. L. Murphy | 24:209-16 |
| PUBLIC HEALTH AND ENVIRONMENTAL MEDICINE | | |
| Sequential Analysis in Therapeutic Trials | P. Armitage | 20:425-30 |
| Smallpox and Smallpox Vaccination Policy | J. M. Lane, J. D. Millar, J. M. Neff | 22:251-72 |
| Use of Physicians' Assistants in the Delivery of Medical Care | E. A. Stead Jr. | 22:273-82 |
| Methadone Treatment of Heroin Addiction | D. X. Freedman, E. C. Senay | 24:153-64 |
| RADIOLOGY AND RADIOACTIVITY | | |
| Technetium-99M in Clinical Nuclear Medicine | A. Gottschalk | 20:131-40 |
| REPRODUCTIVE SYSTEM AND OBSTETRICS | | |
| Fetal Indications for Therapeutic Abortion | A. C. Barnes | 22:133-34 |
| The Prenatal Diagnosis of Inborn Errors of Metabolism | A. Milunsky, J. W. Littlefield | 23:57-76 |
| Estimation of Gestational Age in the Fetal State | J. W. C. Johnson Jr., G. B. Odell | 24:165-74 |
| RESPIRATION | | |
| Assisted Respiration | A. K. Pierce | 20:431-48 |
| Cystic Fibrosis in Adults | S. W. Brusilow | 21:99-104 |
| Alpha1 Antitrypsin Deficiency and Pulmonary Emphysema | C. A. Guenter, M. H. Welch, J. F. Hammarsten | 22:283-92 |
| Clinical Application of Continuous Monitoring of Respiratory Failure | K. R. Tantum, R. D. Dripps | 22:293-304 |

| | | |
|---|---|---|
| Mechanics of Air Flow in Airway Obstruction | A. C. DeGraff Jr., A. Bouhuys | 24:111-34 |
| Endogenous Carbon Monoxide Metabolism | R. F. Coburn | 24:241-50 |